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Novel therapeutics approaches against *Schistosoma haematobium and Opisthorchis viverrini* and associated cancer (bladder cancer and cholangiocarcinoma)

Maria João de Castro Gouveia

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Novel therapeutics approaches against *Schistosoma haematobium* and *Opisthorchis viverrini* and associated cancer (bladder cancer and cholangiocarcinoma)

Tese de Candidatura ao grau de Doutor em Ciências Veterinárias, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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“Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less.”

Marie Curie

To my parents and my sister.
To the memory of my grandmother Aurora.

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LIST OF PUBLICATIONS

Articles already published and submitted and under revision or for publication in peer-reviewed scientific journals, were used in the elaboration of this dissertation. The presentation of each paper in this PhD dissertation does not necessarily reflect a chronological order, since some of the studies described below were done simultaneously.

Publications and manuscripts that depict the results obtained during the thesis and presented in Section III:

- **Gouveia MJ**, Santos J, Brindley PJ, Rinaldi G, Lopes C, Santos LL, Correia da Costa JM, Vale N. Estrogen-like metabolites and DNA-adduct in urogenital schistosomiasis-associated bladder cancer. *Cancer Letters* 2015, 359:226-232.
- **Gouveia MJ**, Pakharukova MY, Laha T, Sripa B, Maksimova GA, Rinaldi G, Brindley PJ, Mordvinov VA, Amaro T, Santos LL, Correia da Costa JM, Vale N. Infection with *Opisthorchis felineus* induces intraepithelial neoplasia of the biliary tract in a rodent model. *Carcinogenesis* 2017, 38: 929-937.
- **Gouveia MJ**, Brindley PJ, Azevedo C, Gärtner F, da Costa JMC, Vale N. The antioxidants resveratrol and *N*-acetylcysteine enhance anthelmintic activity of praziquantel and artesunate against *Schistosoma mansoni*. *Parasite Vectors* 2019, 12:309.
- **Gouveia MJ**, Brindley PJ, Rinaldi G, Gärtner F, Correia da Costa JM, Vale N. Combination anthelmintic/antioxidant activity against *Schistosoma mansoni*. *Biomolecules* 2019, 9:54.
- **Gouveia MJ**, Brindley PJ, Gärtner F, Vale N. Study of effect of antioxidant combined with anthelmintic or anticancer drugs on oviposition of *Schistosoma mansoni in vitro*. (Submitted).
- **Gouveia MJ**, Brindley PJ, Rinaldi G, Gärtner F, Costa JMC, Vale N. Infection with carcinogenic helminth parasites and its production of metabolites induces the formation of DNA-adducts. *Infect Agents Cancer* 2019, 14:41.

- **Gouveia MJ**, Nogueira V, Araújo B, Gärtner F, Vale N. Inhibition of the formation *in vitro* of putatively carcinogenic metabolites derived from *S. haematobium* and *O. viverrini* by combination of drugs with antioxidants. *Molecules* 2019, 24:3842.

RESUMO

As infeções causadas por helmintas são uma das principais causas de morbidez e mortalidade a nível mundial. Ao longo dos anos têm sido desenvolvidas diversas estratégias de controlo com intuito de bloquear a sua transmissão e reduzir a carga parasitária associada às infeções. Apesar disso, a opistorquíase e esquistossomose urogenital (UGS), que foram o foco da presente tese, continuam a ser um problema de saúde pública nos países onde os parasitas são endémicos. Os agentes infeciosos causadores destas infeções, *Schistosoma haematobium* e *Opisthorchis viverrini* são reconhecidos pela Agência Internacional de Investigação em Cancro como causas definitivas de cancro. A infeção causada pelos parasitas origina o desenvolvimento de cancro das células escamosas da bexiga e colangiocarcinoma, respetivamente. No entanto, o mecanismo celular e/ou molecular que associam a opistorquíase e UGS com cancro permanecem desconhecidos. Possivelmente, o desenvolvimento de cancro associado a estas infeções helmínticas é um processo multifatorial. Recentemente, metabolitos de estrogénio e/ou oxiesterois na forma de catecol e os seus respetivos aductos de DNA têm sido implicados, como iniciadores de carcinogénese promovida por estes parasitas. Um dos principais focos da presente tese de doutoramento foi providenciar um conhecimento mais profundo do papel dos metabolitos que derivam dos parasitas no desenvolvimento de cancro associado às infeções causadas por *S. haematobium* e espécies de *Opisthorchis*. Durante o trabalho realizado no âmbito da tese foram obtidas diversas evidências através da utilização de cromatografia líquida acoplada à espectroscopia de massa, que suportam o postulado previamente estabelecido. Evidências essas que incluem a presença de metabolitos de estrogénios, aductos de DNA e derivados de 8-oxodG presentes na urina de 40 pacientes angolanos diagnosticados com UGS; assim como, a presença de diversos metabolitos de oxiesterol semelhante aos descritos para o *O. viverrini* nos estágios de desenvolvimento de *Opisthorchis felineus*, um parasita filogeneticamente próximo do *O. viverrini*, mas considerado não carcinogénico. Por outro lado, evidências da interação dos metabolitos parasitários com DNA do hospedeiro formando aductos de DNA, foram detetadas em amostras biológicas de hamster experimentalmente infetados com *O. felineus*. Ademais, estudos histopatológicos demonstraram que a opistorquíase felinea proporciona um ambiente

celular favorável para o desenvolvimento de um nicho pré-cancerígeno, pelo menos no modelo animal. Nesta tese, também se comprovou a capacidade de compostos semelhantes aqueles descritos para opistorquíase e esquistossomose, em interagir com DNA *in vitro* formando aductos de DNA. A consonância destes resultados parecem suportar a premissa que designa os metabolitos de oxiesterois e/ou estrogénios na iniciação de carcinogéneses através da indução de danos de DNA.

Atualmente, a terapia para estas infeções helmínticas depende de um único fármaco, praziquantel, que apenas têm como alvo o parasita e não as patologias associadas com a infeção. Com intuito de melhorar a atual terapia, desenvolveu-se uma nova estratégia terapêutica baseada na reposição de fármacos e combinação de fármacos com antioxidantes. O principal objetivo desta terapia é não só eliminar os parasitas, mas melhorar as condições patológicas associadas às infeções e em última análise contrariar o processo de carcinogénese. Assim, recorreu-se ao uso de antioxidantes que devido às suas propriedades biológicas poderão ser úteis para inibir a formação de metabolitos putativamente carcinogénicos e bloquear o processo de carcinogénese. Na avaliação da eficácia desta estratégia realizaram-se diversos ensaios *in vitro* utilizando as fases larvais e vermes adultos. O recurso à microscopia ótica e de transmissão eletrónica permitiram observar e quantificar o efeito da nova estratégia terapêutica em eliminar os parasitas. Os resultados demonstraram que não só os antioxidantes por si apresentaram atividade contra as formas larvais e vermes adultos como quando usados em combinação potenciaram a atividade dos fármacos. Adicionalmente, utilizando técnicas de cromatografia líquida acoplada à espectroscopia de massa foi possível realizar estudos relativos ao efeito inibitório da nova estratégia terapêutica na formação de metabolitos previamente associados a esquistossomose e opistorquíase. Com este estudo foi possível demonstrar que combinação de fármaco+antioxidante resulta na inibição quase completa na formação de metabolitos *in vitro* o que pode ser importante no contexto da prevenção da iniciação a carcinogénese associada às infeções mencionadas.

Os resultados apresentados no âmbito da presente tese de doutoramento originaram uma melhor compreensão dos mecanismos moleculares envolvidos na carcinogénese associada a estas doenças helmínticas. Adicionalmente, revelam que a nova estratégia terapêutica, através da combinação fármacos com antioxidantes,

pode ser uma ferramenta valiosa e útil para o combate das mesmas e as suas consequências.

ABSTRACT

Helminth infections are one of the major leading causes of morbidity and mortality worldwide. Several control strategies to block their transmission and reduce the burden associated to infections. Despite that, opisthorchiasis and schistosomiasis urogenital (UGS), which will be our focus, remain a public health problem in endemic countries. The causative agents of these infections, *Schistosoma haematobium* and *Opisthorchis viverrini*, are recognized as definitive cause of cancer by International Agency Research on Cancer: infection with these parasites leads to squamous cell carcinoma and cholangiocarcinoma, respectively. However, the cellular and/or molecular mechanisms linking opisthorchiasis and UGS with cancer remain elusive. Most likely, the development of *S. haematobium* and *O. viverrini* infection-associated cancer is a multistep and multifactorial process. Nonetheless, estrogen and/or oxysterol metabolites in form of catechol and DNA adducts have been implicated, at least in part, as initiators of carcinogenesis promoted by these parasites. One of focus of this doctoral thesis was provided deeper understanding of the role of parasite-derived metabolites in the development of cancer associated with *S. haematobium* and *Opisthorchis* spp. infections. Several evidences by liquid chromatography coupled to mass spectrometer (LC-MS/MS) obtained during the thesis support our previously postulated. Urine analysis of angolan patients demonstrated the presence of estrogen-like metabolites, DNA adducts and derivatives of 8-oxo-dG. Additionally, developmental stages of *O. felineus*, a closely phylogenetic relative of *O. viverrini* considered non carcinogenic, presented several oxysterol-like metabolites similar to those described for *O. viverrini*. Evidences of their interaction with host DNA, e.g. DNA adducts, were observed in biological samples from hamster experimental infection. Moreover, histopathological studies demonstrated that infection with *O. felineus* lead to developmental of a pre-niche cancerous, at least in rodent model, raising question about its carcinogenic potential. In this thesis, we also prove the ability of compounds, similar to those described for opisthorchiasis and schistosomiasis, to interact with DNA *in vitro* leading to formation of DNA-adducts. The consonance of these results seems to support the premise that assign the oxysterol- and estrogen-like metabolites in the initiation of carcinogenesis through DNA damage.

Until now, the therapy against these helminth infections relies on a single drug, praziquantel, that mainly targets the parasite and not the pathologies associated with the infection. In order to improve the current therapy, we developed a novel therapeutic approach based on drug repurposing and combination of drugs with antioxidants. The aim of this novel therapeutic approach is not only eliminating the parasites but also ameliorate the infection-associated pathologies and ultimately counteract the infections-associated carcinogenesis. Thus, the antioxidants were selected due to their biological properties which might be useful to potentially inhibit the formation of putative carcinogenic metabolites and block carcinogenesis. In order to assess its efficacy, we performed several *in vitro* assays either in larval and adult worms. Light and transmission electron microscopy allowed us to observe and quantified the effect of novel therapeutic approach in eliminating the parasite. Not only antioxidant *per se* demonstrated antischistosomal activity against larvae and adult worms but also when combined to drugs enhance their activity. Moreover, using LC-MS/MS was possible to study the inhibitory effect of novel therapeutic approach in formation of metabolites previously associated to schistosomiasis and opisthorchiasis. The results revealed that combination of drug+antioxidant leads to almost a complete formation of metabolites which might be important in context of prevention the initiation of opisthorchiaisis and UGS-associated carcinogenesis.

The results presented in this doctoral thesis open avenues to a better understanding of the molecular mechanisms involved in carcinogenesis induced by opisthorchiasis and UGS. Furthermore, revealed that novel therapeutic approaches that combined drugs with antioxidants might be valuable to combat these helminth diseases and its dreadful consequences.

ABBREVIATIONS

8-oxodG − 8-oxo-7,8-dihydro-2'-deoxyguanosine

ABZ – Albendazole

AIDS – Acquired immune deficiency syndrome

ART – Artemether

AS – Artesunate

BilIN − Biliary intraepithelial neoplasia

Ca^{2+} − ion calcium

CCA – Cholangiocarcinoma

CEQ – Catechol estrogen quinones

CHO – Chinese hamster ovary cells

CI − Combination index

COMT − Catechol-*O*-methyltransferase

CTZ – Clotrimazole

Curc – Curcumin

DALYs − Disability-adjusted life years

DiPept – H-*L*-Tryp-*L*-Ser-OH, Dipeptide

ELISA − Enzyme-linked immunoabsorbent assay

GSH − Gluthatione-S-transferase

FBZ – Flubendazole

FGS – Female genital schistosomiasis

Flav – Flavone

IARC − International Agency for Research on Cancer

IL-6 – Interleukin-6

IMT – Imatinib

INSA – Instituto Nacional de Saúde Dr. Ricardo Jorge

LC-MS/MS – Liquid chromatography coupled to mass spectrometry

MBZ – Mebendazole

MCZ − Miconazole

MDA − Mass drug administration

MEL – Melatonin

MFQ − Mefloquine

NAC − *N*-acetylcysteine

NEM − Newly excysted metacercaria

NDMA − *N*-dinitrosomethylamine

NTD − Neglected tropical diseases

NQO1 − Quinone reductase

NTS − Newly transformed schistosomula

OXA − 4-phenyl-1,2,5-oxadiazole-3-carbonile-2-oxide

PDR − People's Democratic Republic

PZQ − Praziquantel

Resv − Resveratrol

SCC − Squamous cell carcinoma

TBD – Tribendimine

TEM − Transmission electron microscopy

TGR − Thioredoxin glutathione reductase

TMT − Trametanib

UGS − Urogenital schistosomiasis

VDT – Vandetanib

WHO − World Health Organization

XTH − Xanthohumol

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Section I

Introduction and Generalities

I. Introduction and Generalities

1. *Schistosoma* and *Opisthorchis*: threatening helminth parasites

Helminth infections are one of the major leading causes of morbidity and mortality worldwide and are prominent among the so-called neglected tropical diseases (NTDs). It is estimated that almost two billion people worldwide are infected with helminth parasites. These parasites are among the most common infectious agents nowadays and are responsible for several debilitating diseases and syndromes (Hotez et al., 2008; Hotez, 2011; McSorley and Maizels, 2012). Generally, helminth infections are most prevalent in rural communities among tropics and subtropics where sanitation conditions are inappropriate (Hotez, 2011). Despite many efforts to eradicate them, this remains a distant goal due to many factors as a lack to effective treatment, limited pharmacological efficacy, emerging drug resistance, and rapid reinfection in environments where the transmission cannot or is very difficult to interrupt (McSorley and Maizels, 2012). Two important classes of helminths are responsible for the most common and dreadful infections: the nematodes and platyhelminths (also known as flatworms) that include the flukes of genus *Schistosoma* and *Opisthorchis* (Hotez et al., 2008).

1.1. The blood fluke *Schistosoma* and associated infection

1.1.1. Morphology and biology of parasite

The genus *Schistosoma* belongs to the Phylum Platyhelminths, Class Trematoda, Subclass Digenea, Order Schistosomatida and Family Schistosomatidae (Rollinson and Southgate, 1987; Alarcón de Noya and Noya, 2008). Three schistosome species are responsible for the majority of human infections: *Schistosoma mansoni*, *S. haematobium*, *S. japonicum*. Other two species are also able to infect human, *S. intercalatum* and *S. mekongi* (Gryssels et al., 2006; Cook and Zumla, 2009; Colley et al., 2014; Coltart and Whitty, 2015).

The parasites comprehend several developmental stages: eggs, miracidia, sporocysts, cercariae, schistosomula and adult worms. The adult schistosome has a cylindrical tubular elongated body (7-20 mm) that features two rudimentary suckers (oral and ventral), a complex tegument, a blind digestive tract and reproductive organs (Figure I-1). Schistosome tegument, a living syncytium, is one of the most important organs due to its role in protection against the immune attack of the host, nutrients absorption and molecules, and proteins synthesis (Braschi et al., 2006; Skelly and Wilson, 2006; van Hellemon et al., 2006). Parasite produce energy via glucose metabolism from the host through digestion of erythrocytes; which why this helminth is also known as blood fluke (van Oordt el al., 1985; Barret, 2009). The digest system of schistosomes is rudimentary with a blind digestive tract and consequently, they cannot excrete waste products, so they regurgitate them into the bloodstream. These products are useful for blood and urine-based diagnostic assays of schistosome infection as further describe in this section (Colley et al., 2014). Schistosomes are dioecious and not hermaphroditic like the other trematodes (Figure I.1) (Loker and Brant, 2006). Males are shorter and stouter than females and presented a gynecophoral canal in which female lies, thus, adult worms live in copula much of the time (Gryssels et al., 2006; Colley et al., 2014; Coltart et al., 2015). Adult worms are long-lived and can survive in their mammalian host usually 3 to 10 years but in some cases can survive up to 40 years (Colley et al., 2014). During this period the female produces eggs and fertilized them. Similar to energy production, egg rate production is dependent on fatty acid oxidation derived from the host (Huang et al., 2012).

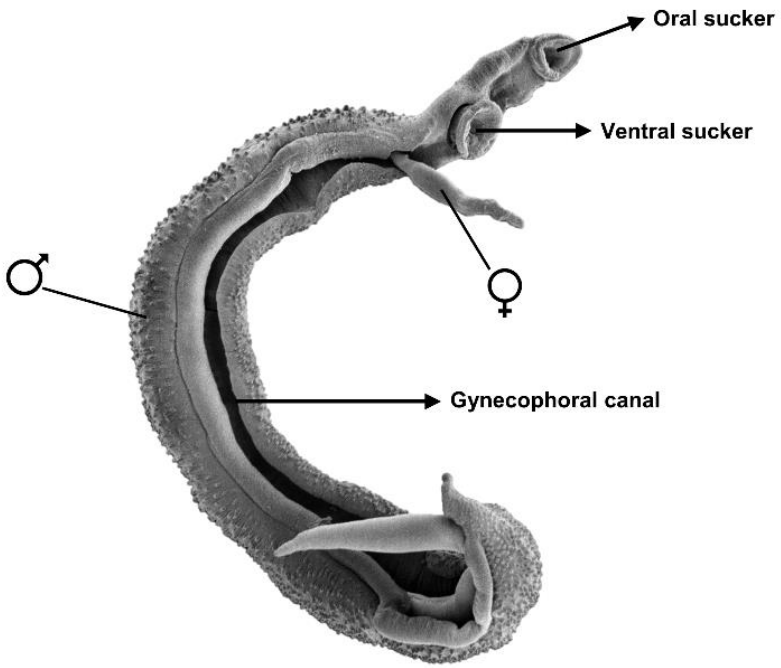

Figure I-1. Adult worms of schistosome coupled. Both worms presented oral and ventral suckers. Female (♀) reside inside gynecophoral canal of male (♂). The tegument of male adult worms is fully covered with spines. (Image adapted from Trustees of the Natural History Museum http://www.nhm.ac.uk consulted on 31st July 2019).

The life cycle of *Schistosoma* spp. is complex involving two hosts: one definitive (humans) and intermediate (snails) (Figure I-2). Along their lifetime, females produce hundreds (African species: *S. mansoni*, *S. haematobium* and *S. intercalatum*) to thousands (oriental species: *S. mekongi* and *S. japonicum*) of eggs per day. The eggs either expelled through urine or feces remain viable up to 7 days or become trapped in the host tissues where they induce inflammation and die. Regardless of whether eggs are excreted or retained in host tissues, they die 1-2 weeks after being released by the female worm. The excreted eggs that reach the freshwater will hatch releasing the miracidium that infects a suitable snail host. Following snail infection, the miracidium undergoes through asexual reproduction from sporocyst stages until forms cercariae, and thousands of them are eventually released from the snail into the water after 4 to 6 weeks. After being released the cercariae can remain infective for 1 to 3 days, however, their energy reserves considerably diminish over a few hours (Colley et al., 2014). Human infection occurs when cercariae, the infectious form for humans and other mammals, contact with the skin during washing or swimming, penetrate it and shed the tail. Afterwards, the juvenile larvae, schistosomula, migrate within the circulatory system until they reach vesicle plexus and veins that drain the ureter and

nearby pelvic organs (*S. haematobium*) or mesenteric veins (all other species), where the worms mature, mate and commence egg laying about 5-7 weeks post-infection (King, 2011; Barsoum et al., 2013; Colley et al., 2014) and thus complete the life cycle. Transmission invariably occurs when schistosome-infected individuals or mammals contaminate freshwater with their urine or feces containing parasite eggs (Gautret et al., 2012). Due to schistosome life cycle, the infection is considered '*the most important water-based disease from a global public health perspective*' demonstrating the importance of water for its spread (Steinmann et al., 2006; Di Bella et al., 2018).

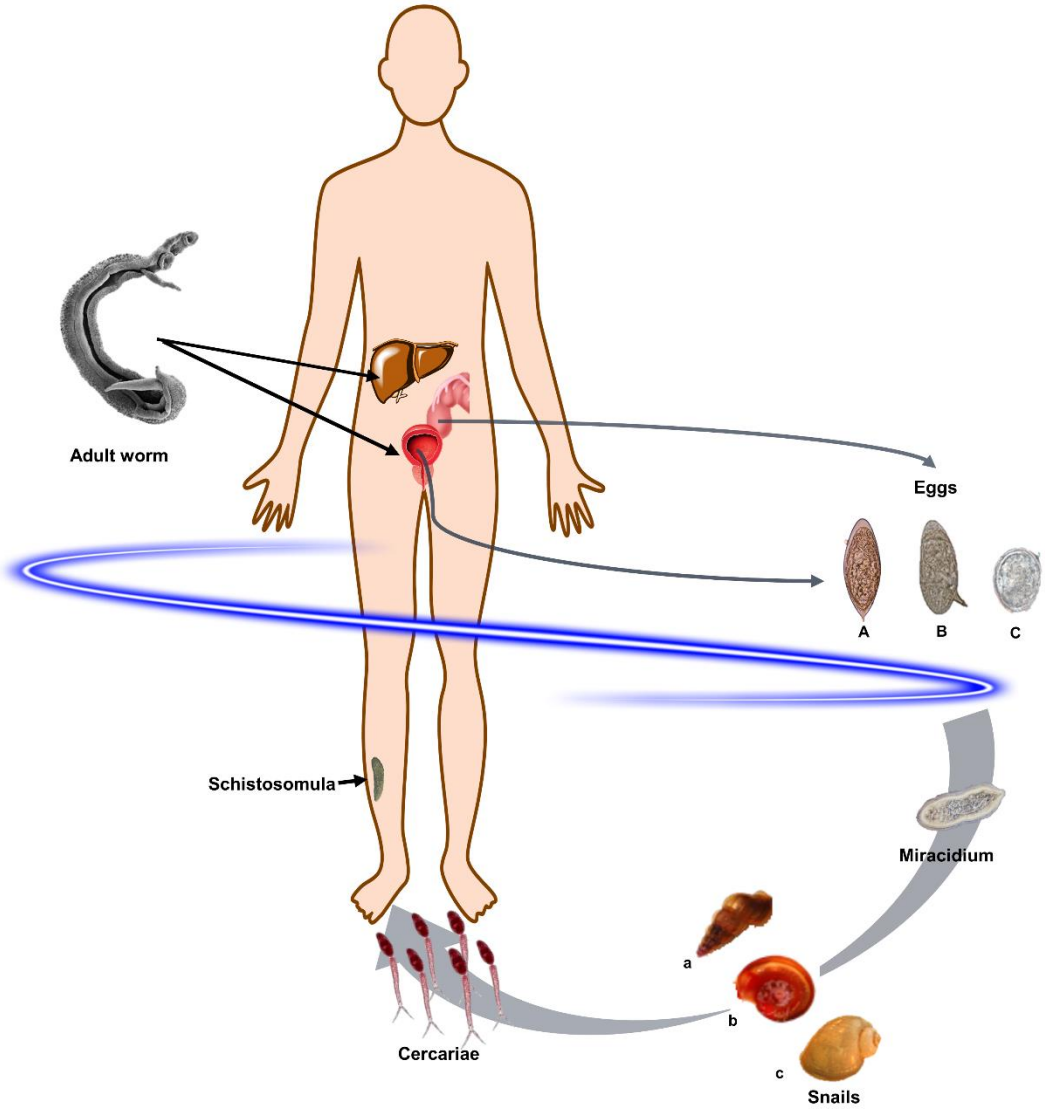

Figure I.2. The developmental cycle of the three main species that infect humans: *S. mansoni*, *S. haematobium*, and *S. japonicum*. The paired adult worms' mate and produce eggs. The egg morphology is useful to distinguish species of schistosomes: *S. haematobium* (A) and *S. mansoni* (B) produce oval eggs with sharp lateral or terminal spine, respectively; *S. japonicum* produce round eggs with a rudimentary lateral spine (C). The eggs also vary slightly in size: *S. mansoni* (115-175 x 45-70µm) and *S. haematobium* (110-170 x 40-70µm) eggs have similar size while *S. japonicum* eggs are smaller (70-100 x 50-70µm). The eggs reach the freshwater release miracidium which infects suitable snails: a) *Onchomelania* for *S. japonicum*; b) *Bioamphalaria glabrata* for *S. mansoni* and c) *Bulinus* for *S. haematobium*. After asexual reproduction the snail release cercariae that when in contact with human skin releases schistosomula which enter to the bloodstream and travel until reach the organ where parasite resides and mature. (Image adapted from Vale et al., 2017a).

The parasite *Schistosoma* not only infect humans, but some species as *S. japonicum* and *S. mekongi* are also zoonotic and can infect several other mammals including dogs, cattle, or pigs. Despite species as *S. mansoni* and *S. haematobium* are

capable of infect animals as rodent and non-human primates, human beings are considering its predominant mammals' hosts. To successfully control and elimination of the human schistosomiasis it is crucial to comprehend the life cycle and the parasite's movement between the intermediate and definitive host (Colley et al., 2014).

1.1.2. History of schistosomiasis

At least for 6000 years ago, there is evidence of infection caused by schistosomes. It remains unclear where the helminth parasite first emerged. Some studies in northern Syrian demonstrated evidence of the presence of this helminth on human skeletal of farmers dated with 5800-4000 years before Christ (Anastasiou et al., 2014). Nonetheless, it has been suggested that helminth first appeared in African areas containing great lakes essential for maintaining the life cycle (Steinmman et al., 2006). The earliest description of schistosomiasis can be found in the ancient Assyro-Babylonian literature, namely in the Papyrus Ebers of Egypt (Girges, 1934) where it is mentioned a worm disease might cause urinary bleeding. However, only in 19th-century efforts were made to understand the life cycle of helminth and the pathogenesis associated with infection (Di Bella et al., 2018). The first description of *Schistosoma* spp. is attributed to German pathologist Theodor Bilharz in 1851. During the autopsy of a young Egyptian soldier, the pathologist found worms in portal veins and described the presence of eggs with a terminal spine (Bilharz, 1853a). These observations suggested that soldier could be infected with *S. mansoni* or *S. haematobium* since eggs of two species share this characteristic (Figure I-2). This finding was reported to his professor von Freholdtz and later presented in a meeting at Breslau where the pathologist described the characteristics pathologic changes and clinical features of schistosomiasis and named the worms as *Distomium haematobium* (Bilharz, 1853b and 1856). In 1858, Weinland disagreed with the nomination and suggested altering the name to *Schistosoma* where '*Schisto*' means cavity and '*soma*' means the body that envelops the female. Two years later, Cobbold (1959) suggest change the designation of infection to bilharzia in honor of Theodor Bilharz. Since 1964, the official nomenclature to infection caused by schistosome is schistosomiasis, however, in Europe and the Middle East, the term bilharzia is also commonly used (Olds and Dasarathy, 2001).

Over the following years and until nowadays, many researchers focused on schistosomes and its infection leading to a deeper understanding of the parasite's life cycle, pathology, and novel *Schistosoma* species.

1.1.3. Schistosomiasis: an overview

The World Health Organization (WHO) considers schistosomiasis one of the major NTD that mainly occurs in tropical and sub-tropical areas (Gryssels et al., 2006; Colley et al., 2014; McManus et al., 2018). Human schistosomiasis occurs mainly through infection of three schistosome species: *S. haematobium* induces urogenital schistosomiasis (UGS), while *S. mansoni* and *S. japonicum* lead to intestinal schistosomiasis. Infections caused by *S. intercalatum* and *S. mekongi* are only for local importance and also causing intestinal schistosomiasis (Gryssels et al., 2006). The species are distributed worldwide (Table I-1 and Figure I.3), and its distribution depends mainly on the ecology of snails hosts and their infection (Colley et al., 2014).

Table I.1. *Schistosoma* species and regions of prevalence of schistosomiasis.

Species	Region of prevalence	Forms of schistosomiasis
S. mansoni	Africa, Middle East, Caribbean, Brazil, Venezuela and Suriname	
S. japonicum	China, Indonesia, Phillipines	
S. mekongi	Several districts of Cambodia and Laos People's Democratic Republic	Intestinal schistosomiasis
S. intercalatum	Rain forest areas of central Africa	
S. haematobium	Africa, Middle East, Corsica (France)	Urogenital schistosomiasis

Based on update and correct data from: (https://www.who.int/en/news-room/fact-sheets/detail/schistosomiasis - last access on July 31st 2019).

Over 250 million people are infected with schistosomes worldwide, with 201.5 million (97% of cases) of them live in Africa (Steinmann et al., 2006, Utzinger et al., 2009; Hotez et al., 2014). About half of all infected people have symptoms with 20 million suffer from severe consequences. It is estimated that schistosomiasis causes

about 280 000 deaths annually and 779 million people or more are at risk of infection in 76 countries (Figure I.3) (Steinmman et al., 2006; Utzinger et al., 2009; WHO, 2013). According to the Global Burden of Disease Study 2016, the global burden of schistosomiasis is estimated at 1.9 million disability-adjusted life years (DALYs) (GDB, 2017), however, prior studies estimated the global burden of schistosomiasis at 1.7-4.5 million DALYs which suggest that estimative might be underrated (WHO, 2002; Utzinger and Keiser, 2004).

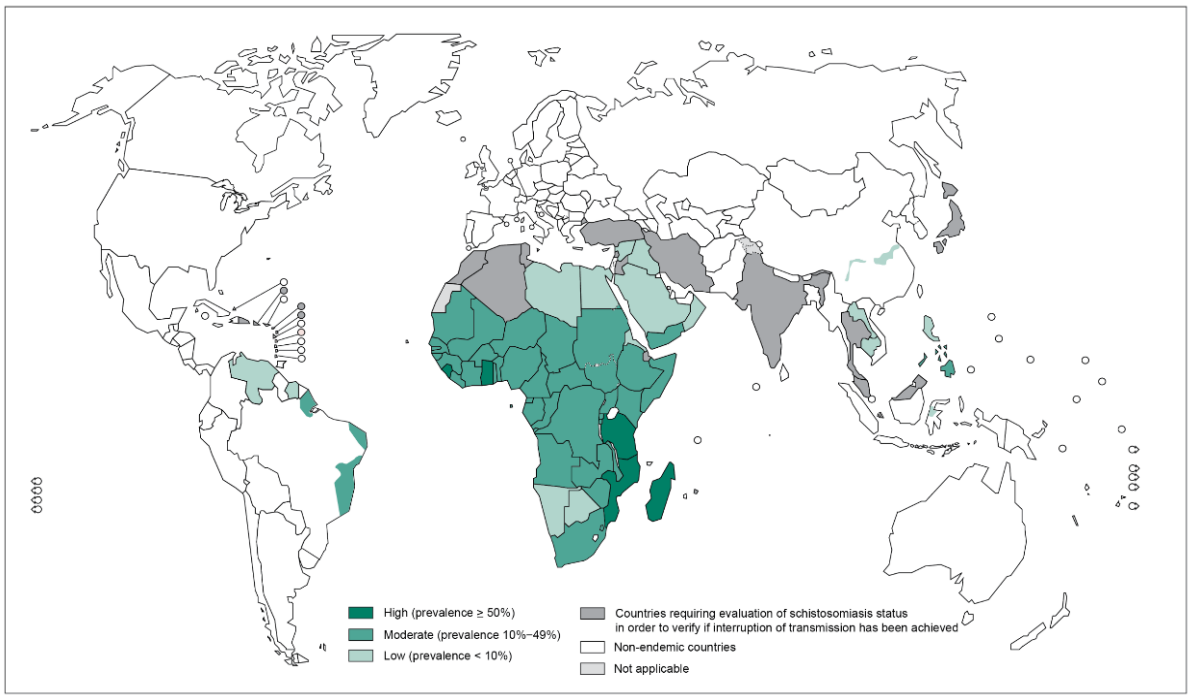

Figure I.3. Worldwide distribution of schistosomiasis considering the prevalence rate. Most of cases occurs on the African continent. (Figure from Map: Distribution of schistosomiasis, worldwide, 2012, WHO, © 2012).

Although schistosomiasis is generally restricted to the tropics and sub-tropics, several cases of infection have been reported in Europe. Most of them occur due to the arrival of infected immigrants and in returning travelers from countries where the infection is endemic (Lingscheid et al., 2017; Marchese et al., 2018). Between 1997 and 2010, more than 1465 cases of imported schistosomiasis have been reported by the European Network for Tropical Medicine and Travel Health (Lingscheid et al., 2017). Notably, the recent cases reported in Corsica are not related to immigrants or travels (Holtfreter et al., 2014; Boissier et al., 2015) suggesting the expansion of suitable habitats for transmission of schistosomiasis and its potential of reemerging in economically developed regions in southern Europe (Holtfreter et al., 2014).

Furthermore, recent studies point to the potential hybridization of species originating two novel hybrid parasites from hybridization between *S. haematobium* and *S. mansoni* (Le Govic et al., 2019) and species of *S. haematobium* and *S. bovis* (bovine blood fluke) (Moné et al., 2015; Oey et al., 2019).

1.1.3.1. Urogenital schistosomiasis: clinical presentation

Considering that *S. haematobium* resides in the urinary tract, the infection caused by parasite is designated urogenital schistosomiasis (UGS). Generally, the progression of schistosomiasis comprehends three general stages: acute, established active and chronic infection (McManus et al., 2018). The stages differ in egg excretion rates in urine as well as clinical manifestations and symptoms (McManus et al., 2018).

The acute phase is typically characterized by an acute, pruritic, maculopapular eruption, cercarial dermatitis, at the site of cercarial skin penetration within the first 24h (Chofle et al., 2014; McManus et al., 2018). Cercarial dermatitis occurs due to a portion of the infectious larvae that die in the skin which triggers the innate immune response and give rise to hypersensitivity reactions. Following successful cercariae penetration and maturation, the infection may proceed to a symptomatic stage also known as Katayama fever or Katayama syndrome. The symptoms are caused by systemic hypersensitivity reactions and formation of immune complexes in response to antigens released during schistosomula migration and initiation of egg deposition. Most commons symptoms are fever, myalgia, malaise and fatigue, headache, non-productive cough, and intestinal symptoms as abdominal tenderness or pain which usually appear between 2 weeks and 3 months after exposure. Interestingly, acute schistosomiasis is rarely observed in people living in *S. mansoni* or *S. haematobium*-endemic areas (Gryssels et al., 2006 and 2012; Colley et al., 2014; Chofle et al., 2014; McManus et al., 2018). Following the initial stage, the disease reaches a stage of established active and chronic infection, with mature adult worms and well-established egg production. The symptoms and pathological alterations of chronic infection are closely associated with the passage of parasite eggs through the urinary bladder wall and egg deposition in the bladder (Gray et al., 2011). It is usually finding a cluster of living eggs in bladder tissues that induce intense inflammatory reaction and eosinophilia (Randrianasolo et al., 2015). This inflammatory reaction is required to aid the excretion of eggs to the environment, and the passage of eggs into the lumen of

the bladder commonly induce hematuria. Additionally, can result in bladder wall thickening and development of masses and pseudopolyps, inflammation, granulomas, acute and chronic pyelonephritis, hydrophenosis, kidney malfunctions, among others (Burki et al., 1986; Hatz et al., 1990; Kayange et al., 2014). During this stage, eggs are also frequently deposited in genital organs like the cervix, seminal vessels and prostate leading to possible lesions and inflammation (Leutscher et al., 2000; Kjetland et al., 2006). Most of the women infected with *S. haematobium* also suffer from female genital schistosomiasis (FGS) resulted from egg deposition in uterus, vulva, and vagina and consequently triggers an inflammatory response that affects fertility (King, 2018). In men and female, the manifestation of UGS may increase HIV transmission (Ketjland et al., 2006). Generally, during the late chronic infections little or no detectable eggs are excreted, however, occurs accumulation of dead calcified tissues eggs which appear as yellow sandy patches in the cystoscopic examination of the bladder mucosa (Burki et al., 1986).

1.1.3.2. Diagnostic of urogenital schistosomiasis

The gold standard for UGS diagnosis is based on detection of schistosome eggs in urine and tissue biopsy samples. Briefly, the collected urine, usually a small amount, is filtered and the eggs in that volume are counted microscopically. The level of infection is expressed as eggs per 10 ml of urine (Katz et al., 1972; Feldmeier et al., 1982 and 1986). However, in light infections and late chronic stage of infection, the method has low sensitivity (Feldmeier et al., 1986). Therefore, it is necessary to use alternative methods including serology for detection of schistosome antigens and anti-schistosome-specific antibodies, and polymerase chain reaction (PCR) for detection of worm DNA. These methods increase the sensitivity up to 90% and allow detect the infection during the acute phase and low burden of infection (Van Lieshout et al, 1997; van Dam et al., 2013; Meurs et al., 2015; He et al., 2018; Grenfell et al., 2018).

Besides laboratory techniques, immunological diagnosis is a valuable complementary tool that has been used in population-based studies for >20 years. Ultrasonography can be used to visualize pathological changes and monitoring the direct effect of interventions on morbidity associated with infection. Nowadays, standardized protocols that allow classified and grade the pathological lesions are already available (Barata et al., 1999; Chigusa et al., 2006).

1.1.4. Biological carcinogenic aspect of *Schistosoma haematobium*

About two-thirds of the 90% of schistosomiasis in Africa are caused by *S. haematobium*, the only *Schistosoma* specie considered as group 1 biological carcinogenic by International Agency for Research on Cancer (IARC) (IARC, 2012).

The infection caused by *S. haematobium*, UGS, is associated with the risk of development of squamous cell carcinoma (SCC) of the bladder (Vennervald et al., 2009, IARC 2012). Indeed, bladder cancer is a frequent and dire complication of chronic UGS. Bladder cancer may be a consequence of many years of exposure to parasite, infection and urinary tract inflammation (Vennervald et al., 2009) due to continuous oviposition by the parasite (McManus et al., 2018). As mentioned, parasite eggs excrete several metabolites to transverse the bladder, yet, about half the eggs remain trapped on vesical mucosa causing inflammatory responses that not only conducted to haematuria but other serial clinical conditions such as urothelial dysplasia, hyperplasia and ultimately bladder cancer (Honeycutt et al., 2014). Case reports indicate that patients with UGS may develop bladder cancer earlier than uninfected people (Mostafa et al., 1999; Herrera et al., 2005; Porta et al., 2011). However, the cellular and/or molecular mechanism linking *S. haematobium* infection with carcinogenesis remain elusive. Further, in this section, it will be discussed the possible mechanisms of carcinogenesis associated with UGS.

1.2. Liver fluke *Opisthorchis viverrini*: infection and carcinogenic potential

1.2.1. Morphological and biological aspects of parasite

Opisthorchis spp. are liver fluke parasites that also belong to the Phylum of Platyhelminths and class Trematoda but differ from schistosomes on subclass (Digenea), family (Opisthorchiidae), and genus (Opisthorchis). *Opisthorchis viverrini* and *O. felineus*, also known as the Southeast Asian liver fluke and as the cat liver fluke, respectively, are epidemiologically significant species (Beaver et al., 1984). These parasites are zoonotic and affect both human and animal liver and bile ducts (Keiser and Utzinger, 2005; Petney et al., 2013; Pakharukova and Mordvinov, 2016). Similar to schistosome species, opisthorchiids comprehends several developmental stages including eggs, miracidia, sporocysts, cercariae, metacercariae and adult

worms (Utzinger and Keiser, 2005). The adult forms present a flat body, leaf-shaped, transparent within about 10-25 mm in length and 3-5 mm in width, covered with a noncellular cuticle, two oral suckers, excretory bladder, and reproductive organs (Figure I-4) (Sripa et al., 2008). These parasites are hermaphrodite flukes that reproduce by self-fertilization (Yossepowitch et al., 2004). They have two testicles situated near the posterior extremity and a multilobate ovary in front of the anterior testicles (Ash and Oreil, 2007). The lifespan of adult worms is up to 25-30 years and during that period they produce an estimated 50–200 eggs/g of feces/day (Sithithaworn and Haswell-Elkins 2003; Sripa and Pairojkul, 2008). Eggs are ovoid with a yellowish-brown colour and have a distinct operculum, which opens to release the miracidium, a fully developed larva. In average, eggs are 19-30 μm long by 10-20 μm wide (Sithithaworn and Haswell-Elkins 2003).

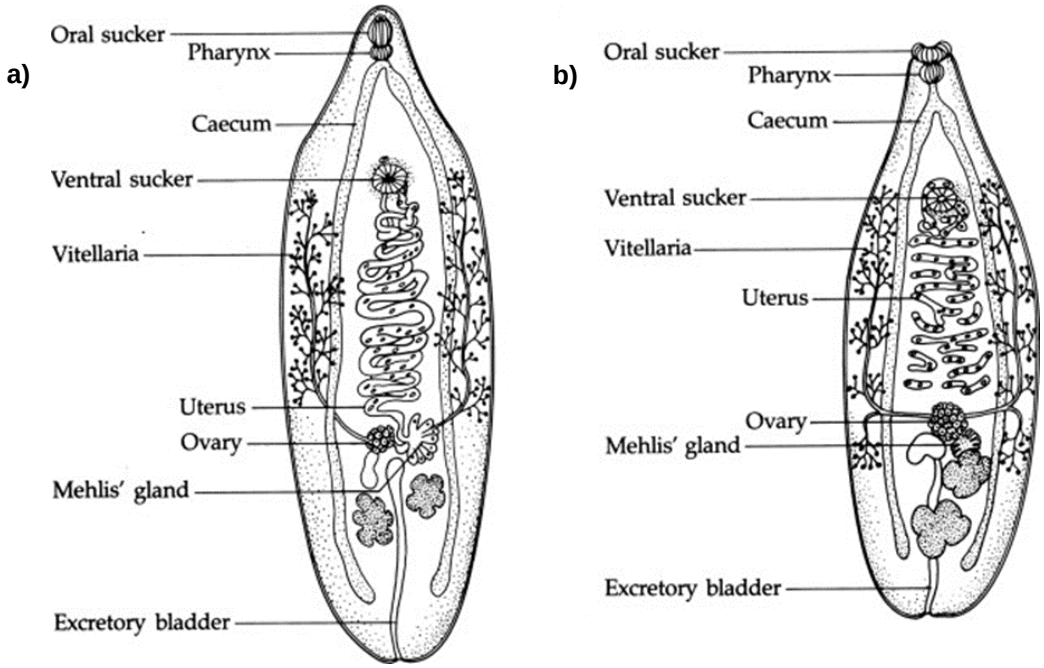

Figure I-4. Morphology of adult worms of a) *Opisthorchis felineus* and b) *Opisthorchis viverrini* (Figure reproduced from Bogitsh et al., 2013).

For energy production the parasite uses glycolysis, yet, the glucose levels are reduced in bile (Masyuk et al., 2002). Therefore, the parasite evolved to adapt to a life there that allowed the degradation of free lipids and proteins to obtain energy (Young et al., 2014).

The *Opisthorchis* spp. have a complex life cycle involving two intermediates hosts, snails of genus *Bithynia* and cyprinid fishes, and definitive fish-eating mammals including cat, dogs, pigs, and accidentally, humans (Figure I-5) (Petney et al., 2013).

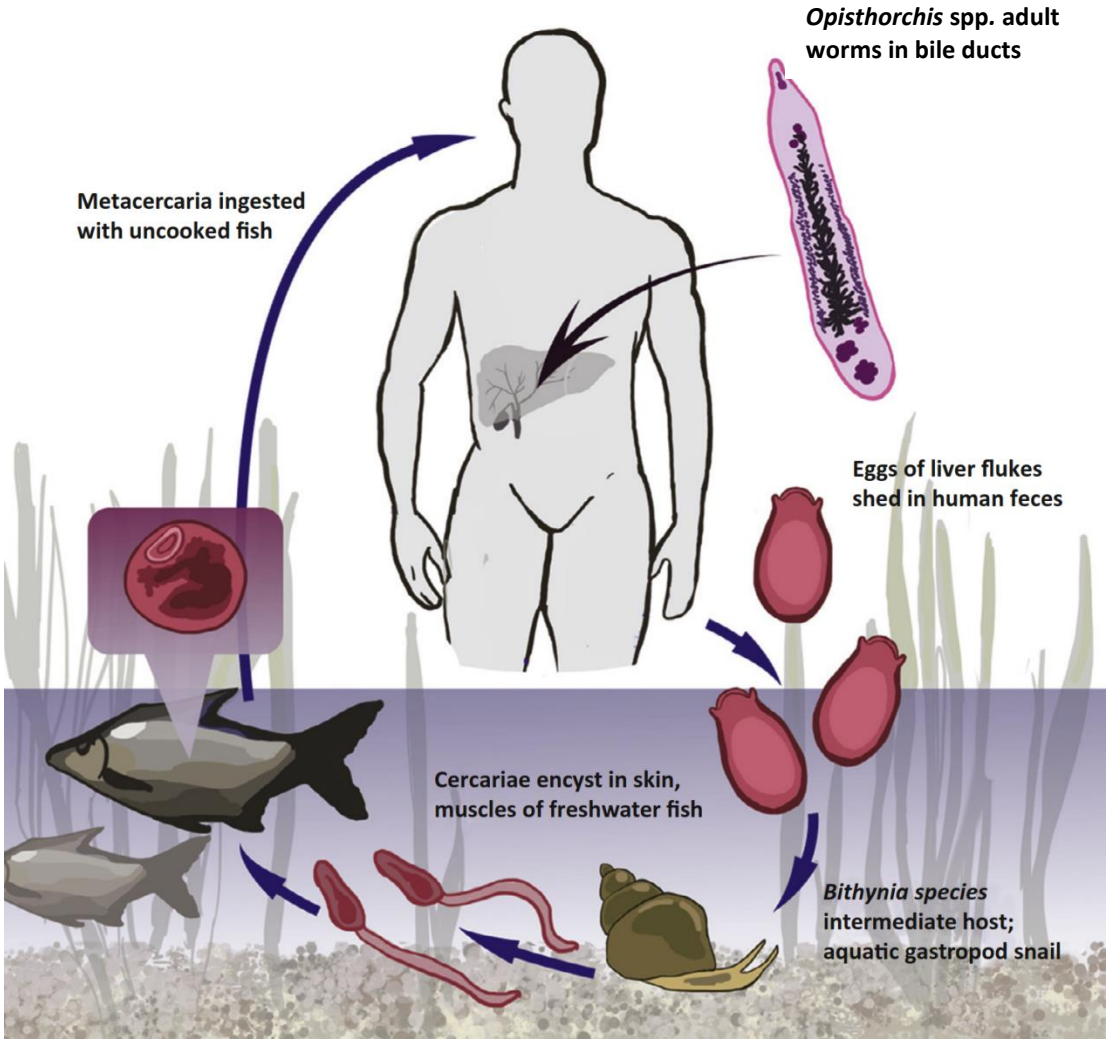

Figure I-5. Developmental cycle of the liver flukes *Opisthorchis viverrini* and *Opisthorchis felineus*. Adult forms shed eggs that pass with bile into the small intestines, and then are excreted through feces. These eggs are ingested by snails in a situation where human waste from infected people is deposited into the freshwater environment. Following ingestion, the parasite's eggs hatch and miracidium undergoes through asexual reproduction that culminates in the release of cercariae into freshwater. The cercariae seek suitable species of fish of the family Cyprinidae where encyst. When mammals eat contaminated raw or undercooked fish, the metacercariae release the juvenile worm in the duodenum, which migrates until reaching the bile duct tree, where mature and produces eggs, completing the life cycle (Figure reproduced from Brindley et al., 2015).

The eggs shed by adult worms are deposited in the biliary tree of the definitive host. Follow that, they reach the intestine with bile and are excreted through feces. Eggs

that reach water release the miracidia that are ingested by snails where undergoes through asexual reproduction to sporocysts, rediae, and cercariae for 4-5 weeks. Then, the cercariae are released into the water, seeks for fish and penetrate the skin between their scales. After a few days in the fish muscle, cercariae encyst as metacercariae, the infective form of the parasite. Animals and humans are infected through digestion of raw or undercooked fish (Kaewpitoon et al., 2008; Sripa et al., 2011), a common traditional practice in the countries where *Opisthorchis* spp. is endemic (Grundy-Warr et al., 2012). Following ingestion, metacercariae excyst in the duodenum and migrate through the ampulla of Vater into bile ducts of the liver, intrahepatic or extrahepatic ducts, where they mature into adult worms over a period of four weeks and start release eggs. In heavy infections, adult worms can also be found in gallbladder (Kaewpitoon et al., 2008; Sripa et al., 2010, 2011). The closely related liver fluke *Opisthorchis felineus* has a similar life cycle (Sripa et al., 2010).

1.2.2. Historical review of opisthorchiasis

The first description of parasites from *Opisthorchis* genus was performed by Rivolta in 1884 following the discovery of fluke in a cat liver from Nothern Italy. Initially, Rivolta (1884) classified the parasite as *Distoma felineus*. A few years later in 1891, a Russian scientist Vinogradov discovered this parasite in human and named it the "*Siberian liver fluke*". Later, the parasite was renamed as *Opisthorchis felineus* which derived from Greek opisthen (behind) and orchis (testicles) (CDC, 2014). The parasite was firstly described in northern Thailand in 1911 during the autopsy examination of two prisoners (Leiper, 1915). Afterward, it was reported that 17% of 230 adult male prisoners examined in the same prison that Leiper conducted autopsy, were infected with was though to be *O. felineus* (Kerr, 1916). In 1927, a decade later, the worms were identified in young Thai male residing in the Northeast of Thailand (Prommas, 1927). During that decade, it was reported the finding of *O. viverrini* among Laotian people in Vientiane (15%) and Takhek (23%) (Bedier and Chesnau, 1929). A year after, the life cycle of *O. felineus* was described by german helminthologist Hans Vogel (Be'er, 2005). During the following decades of 50 and 60s, several researchers contribute to further knowledge of liver fluke infection in Thailand (Sadun, 1955; Harinasuta and Vajrasthira 1962a, b; Wykoff et al., 1965). In 1955, Sadun stated that liver fluke infection observed

in Thailand was caused by *O. viverrini*, not by *O. felineus*, as first hypothesized, which was confirmed a decade later by Wykoff (1965).

1.2.3. Opisthorchiasis: from geographical distribution to clinical presentation

The term opisthorchiasis indicates two distinct infections: opisthorchiasis viverrini and opisthorchiasis felinea which are caused by *O. viverrini* and *O. felineus*, respectively. The geographical distribution of opisthorchiasis depends on two major factors: 1) the presence of the adequate intermediate hosts, snails, and fishes; and 2) consumption of raw or undercooked fish by local people (Sripa et al., 2011). The parasites have different geographical distribution which sometimes might overlap (Figure I.6).

Usually, most of *O. viverrini* infections occur in countries bordering the Mekong River in Southeast Asia (Khuntikeo et al., 2018). Opisthorchiasis viverrini is highly prevalent in Thailand and Lao People's Democratic Republic (PDR) affecting an estimate of 10 million people of these countries, but also occurs in Cambodia and Vietnam (Jongsuksuntigul and Imsomboon, 2003) (Figure I-6a). Recently, cases of opisthorchiasis viverrini in Myanmar have been reported (Aung et al., 2017).

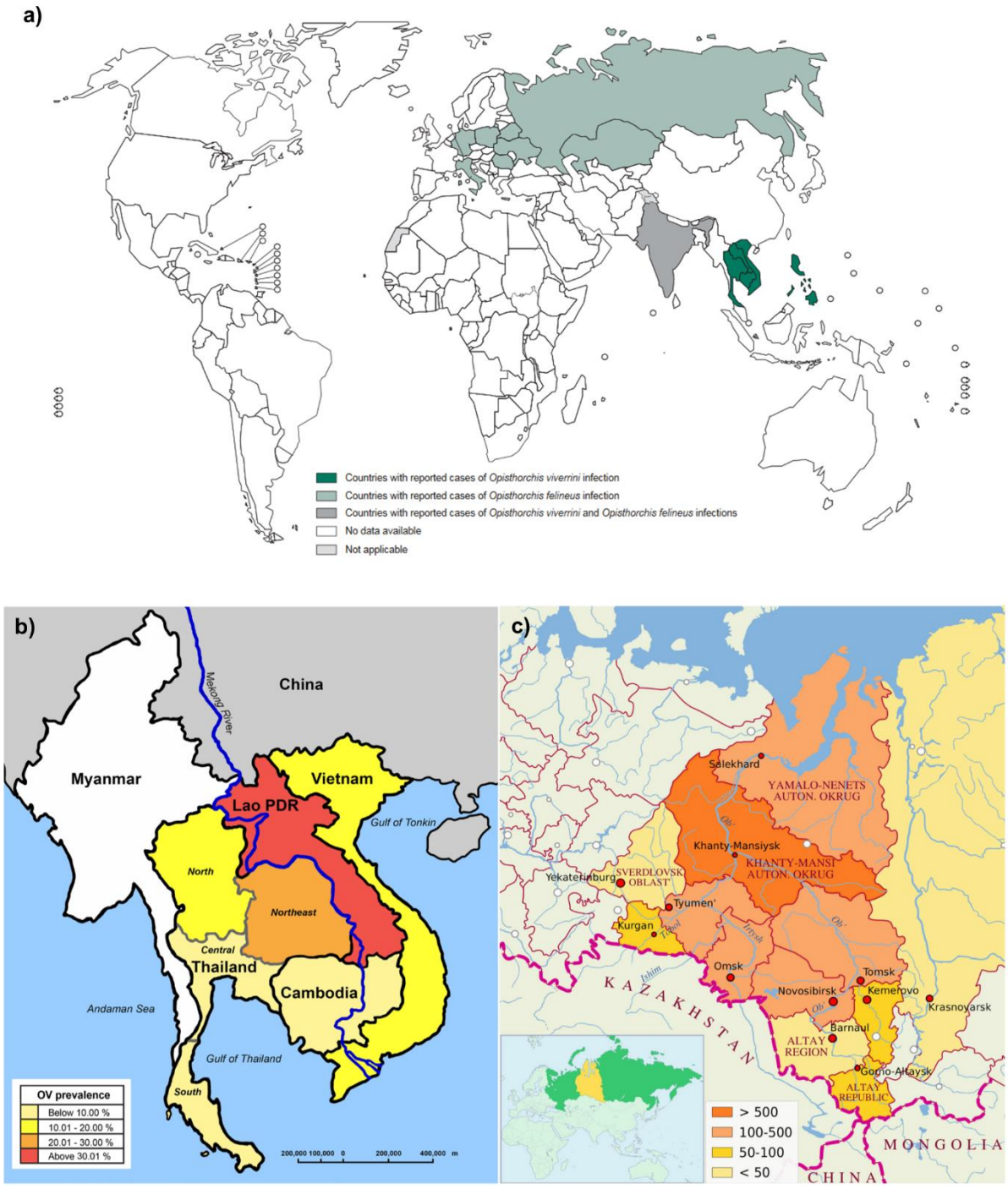

Figure I-6. a) Global geographical distribution of *Opisthorchis viverrini* and *Opisthorchis felineus* infections. Occasionally, the geographical distribution of the two species overlapps (Figure reproduced from https://www.who.int/foodborne_trematode_infections/Distribution_of_opisthorchiasis_worldwide_1280x876px.png. Last access on 6th August 2019); **b) Distribution of *Opisthorchis viverrini* in Southeast Asia** (Image reproduced from Khuntikeo et al., 2018)**; c) Prevalence of *Opisthrochis felineus* in Western Siberia which is higher than other part of Russia** (Image reproduced from Pakharukova and Mordvinov, 2016).

In Thailand, the prevalence rate of infection differs in the four regions of the country with 80% of cases occurring in the North and Northeast regions (Figure I-6b) (Jongsuksuntigul and Imsomboon, 2003). In 2014, the prevalence rate had decrease leading to a corresponding decline in the national prevalence to 5.2% (Wongsaroj et al., 2014). Probably, this decrease occurred due to intensive and continuous control activities over the decades (Suwannatrai et al., 2018). Nevertheless, the prevalence of infection in many individuals' providences within the North and Northeast and at the community level remains elevated (Wongsaroj et al., 2014; Suwannatrai et al., 2018). In Lao PDR the infection is widespread, and it is estimated that more than half population along Mekong River is infected (Rim et al., 2003; Parkin et al., 2006; Sayasone et al., 2007). The elevate prevalence is most likely related to the practice of consuming raw fish in communities in rural providence where up to 60% of fish carry infective form of parasite, metacercariae (Sayasone et al., 2007). On another hand, 30% of domestic companion animals of those communities were infected with *O. viverrini* contributing to increase transmission of the parasites (Xayaseng et al., 2013). The number of cases of opisthorchiasis in Cambodia and Vietnam is unknown (Shin et al., 2010).

The closely relative *Opisthorchis felineus* is highly endemic in Ukraine, Belarus, Kazakhstan, the Baltic countries and especially in Western Siberia where the percentage of infection varies from 10 to 45% (Figure I-6c) (Marcos et al., 2008; Pakharukova and Mordvinov, 2016). Of 17 million people infected with *Opisthorchis* flukes, it is estimated that 1.6 million are cases of opisthorchiasis felinea (Pakharukova and Mordvinov, 2016). In the European Union, human opisthorchiasis felinea was diagnosed more than 50 years ago in Germany, Greece, Lithuania, Poland, Romania and Spain (Erhardt, 1962). Nowadays, sporadic human infections have been documented in Germany and Greece (Sänger et al., 1991; Tselepatiotis et al., 2003). However, the parasite has been detected in red foxes, cats, dogs, fish and mollusks of several European countries as Germany, Poland, Portugal, Spain, Greece and Italy (Hering-Hagenbeck et al., 1996; Oliveira et al., 2005; Pozio et al., 2013). In the last years, outbreaks of human infection with *O. felineus* have been reported in Italy (Armignacco et al., 2008, 2013; Traverso et al., 2012; Pozio et al., 2013). From 2003 to 2011, a total of 211 cases of human opisthorchiasis felinea have been confirmed in Italy (Pozio et al., 2013). The changes on food habits with increasing consumption of

raw marinated fish, among increase of travelers and/or migrants flow through endemic-non endemic countries might turn opisthorchiasis felinea in an emerging parasitic disease in Europe (Pozio et al., 2013).

1.2.3.1. Pathology and pathogenesis

The infection caused by *Opisthorchis* spp. is associated with several hepatobiliary diseases (Mairiang and Mairiang, 2003; Yossepowitch et al., 2004). The pathological and clinical consequences of opisthorchiasis are related to the intensity and duration of infestations. Most of the infections caused by *O. viverrini* are asymptomatic. Usually, there is no acute phase, and only 5-10% of heavily infected individuals present mild symptoms like dyspepsia, abdominal pain, constipation or diarrhea (Upatham et al., 1984; Mairiang and Mairiang, 2003; Kaewpitoon et al., 2008). During the chronic phase of infection, the symptoms can be more severe inducing several pathologic changes in liver, gall bladder, and extrahepatic bile ducts whereas parasite resides as adult form. For example, the biliary ducts might suffer a mechanical injury due to either feeding and migration of flukes, hook of oral and ventral suckers onto the biliary epithelial, release of metabolic products by parasite from tegument and excretory opening that come into contact with the bile duct epithelium. These metabolic products are thought to be immunogenic, mitogenic, and could be toxic. Consequently, their contact with biliary epithelium might origin cell desquamation, hyperplasia, periductal and periportal fibrosis, cholangitis, cholecystitis, and ultimately to the development of cholangiocarcinoma (CCA) (Sripa et al., 2007). Commonly, the liver and bile ducts of infected individuals are enlarged and dilated showing a prominent fibrotic wall (Hitanant et al., 1987; Riganti et al., 1988; Sripa, 2003), also subsequent cystic and saccular formations might occur (Harinasuta and Bunnang, 1990). Similar damage can occur to gallbladder which ultimately becomes unfunctional (Harinasuta and Bunnang, 1990).

Opisthorchiasis felinea is associated with a more pronounced and severe acute phase than opisthorchiasis viverrini. Acute opisthorchiasis felinea may be also asymptomatic, however, if the number of worms was significant symptoms as high-grade fever, malaise, anorexia, diarrhea or constipation, arthralgia, lymphadenopathy, urticarial skin rash, dull pain and discomfort in the upper right quadrant of the abdomen are not infrequent. These symptoms may be associated with intermittent colic pain

caused by obstruction of the gallbladder by the worms. The chronic infection with *O. felineus* induced similar clinical manifestation and pathologies described for *O. viverrini* (Lvova et al., 2012; Maksimova et al., 2017). During this stage, it is commonly observed periductal fibrosis, destruction of the adjacent liver parenchyma, suppurative cholangitis, liver abscess, and granulomas (WHO, 1995b; Yossepowitch et al., 2004). Curiously, comparative studies of opisthorchiasis viverrini and felinea in a hamster model suggest that the histopathology of *O. felineus* infection might be more severe than of *O. viverrini* (Lvova et al., 2012). Also, *O. felineus* physiology, molecular biology, mechanism host-parasite interaction, and carcinogenic potential are less well studied than *O. viverrini* (Maksimova et al., 2017).

1.2.3.2. Diagnosis of opisthorchiasis

The gold standard for diagnosis of both opisthorchiasis is the fecal examination of the fluke egg's (Sithithaworn et al., 1991; Upatham et al., 2003; Yossepowitch et al., 2004; Sripa et al., 2011). The sensitivity and specificity of parasitological diagnosis rely on examination and experience of the microscopist. Moreover, in cases of light infection or bile duct obstruction, eggs may not be detected in the feces and might lead to a false negative result (Sithithaworn et al., 1991). Thus, there is a need for a reliable diagnostic tool to overcome these issues (Yossepowitch et al., 2004). Several serologic/immunological tests for the diagnosis of opisthorchiasis including the intradermal test, immunoelectrophoresis, indirect haemagglutination assay, indirect fluorescent antibody test and indirect enzyme-linked immunoabsorbent assay (indirect ELISA) have been reported within greater sensitivity and specificity than fecal exams. The indirect ELISA is favoured for its ease and the increased sensitivity and specificity of diagnostic obtained by an antibody-based system (Srivatanakul et al., 1985; Thammapalerd et al., 1988; Sirisinha et al., 1991; Chaicumpa et al., 1992; Sirisinha et al., 1995; Watthanakulpanich et al., 1997; Wongsaroj et al., 2001; Waikagul et al., 2002). Nevertheless, the complex composition of the crude antigen extracts and some cross-reactions with other parasitic infections might constitute a drawback of serological tests (Yossepowitch et al., 2004). More recently, molecular techniques as DNA hybridization and PCR-based methods have been investigated. However, further evaluations on their sensitivity and specificity are still required before its potential implementation (Wongratanacheewin et al., 2001, 2002; Ruangsittichai et al., 2006).

Despite the techniques mentioned above might be very effective for diagnosis of opisthorchiasis, so far, none of these methods replace the microscopic examination (Upatham et al., 2003). Imagiological tools as ultrasound, computed tomography or magnetic resonance imaging scans for diagnosis of advanced periductal fibrosis due to dilation and thickening from fibrotic deposition is already available (Mairiang et al., 2006).

1.2.4. *Opisthorchis* spp., major risk for development of cholangiocarcinoma

Opisthorchis viverrini is also a definitive cause of cancer belonging to Group 1 carcinogens (IARC, 1994). The correlation between the incidence of CCA and the high prevalence of opisthorchiasis viverrini was determined by epidemiological studies (Parkin, 2006; Shin et al., 2010). CCA is the most predominant type of liver cancer in Thailand (Sripa and Pairojkul, 2008), especially in the Northeast where the prevalence of infection is higher as mentioned above. Commonly, the first *O. viverrini* infection occurs in childhood which evolves into chronic infection in residents of endemic areas, and after three to four decades, the liver cancer is diagnosed (Sripa et al., 2011). CCA is a primary liver cancer-derived from cholangiocytes of the extrahepatic and intrahepatic bile ducts. This type of cancer is extremely invasive, develops rapidly, often metastasized, and has a very poor prognosis (Blechacz and Gores, 2008). Usually, it is asymptomatic during the initial phase and most of the times is diagnose in advance stage of cancer, thus, CCA is known as "*silent killer*" (Khuntikeo et al. 2018). The death of patients usually occurring within 3-6 months after diagnostic (Andrews et al., 2008). In northern Thailand, it is estimated that 5000 cases/year of CCA are diagnose (Parkin, 2006), which translates into 5000 deaths annually (Sripa et al., 2011). There is no medical treatment available, and surgery and supportive treatment are complicated due to the location of tumors, and often not accessible for patients in developing countries (Malhi and Gores, 2006).

Unlike *O. viverrini*, its relative *O. felineus* do not belong to biological agent group 1, i.e. is not considered as carcinogenic, but is classified as potentially dangerous for humans (Group 3) (IARC, 2012). Until now, no association between *O. felineus* infection and CCA has been demonstrated. However, the prevalence of CCA in endemic areas of *O. felineus* in Western Siberia is three times higher than in another part of Russia (Sripa et al., 2007; Mordvinov et al., 2012). The role of parasite in the

development of CCA in humans remains elusive, probably due to the scarce of information regarding epidemiological and clinical programs for opisthorchiasis felinea (Pakharukova and Mordvinov, 2016). Nevertheless, the similarity of clinical manifestations and diseases caused by both parasites and the experimental data obtained through hamster model of infection opisthorchiasis felinea (Lvova et al., 2012; Maksimova et al., 2017) suggest that probably *O. felineus* also might have carcinogenic potential similarly to *O. viverrini*. Recent findings highlight the need for reconsideration the classification of IARC regarding infection with *O. felineus* (see Section III-Chapter 2).

The mechanisms responsible for the onset of helminth-infection associated cancers remain unknown, and presumably, derived from a multifactorial process (Sripa and Pairojkul, 2007; Sripa et al., 2010). During the next chapter of this section, it will be discussed how and why helminths as *S. haematobium* and *O. viverrini* can induce cancer-associated to infection.

2. Helminths and infection-associated cancer

Infections are responsible for more than 20% of cancers in the developing world. The infections cause by several agents, mainly virus as Hepatitis B and C, human papillomavirus, *Epstein Barr* virus, and bacteria as *Helicobacter pylori* are widely known and recognized as biological cancers (Pagano et al., 2004; Bouvard et al., 2009; de Martel et al., 2012; IARC, 2012). Less appreciation has been given to helminth infections as a direct cause of malignancy. Since the early 1990s, infections caused by *S. haematobium* and *O. viverrini* have been recognized as the definitive cause of cancer and directly correlate with the development of SCC of the bladder and CCA, respectively (IARC, 1994, 2012). Interestingly, the chronic infection caused by other phylogenetic relatives of these parasites, e.g. *Fasciola hepatica*, has not been proven to be carcinogenic (Brindley et al., 2015). Why some helminths are causative agents of cancer? These differences may be related to several factors and/or a combination of thereof. Either the existence of specific helminth-metabolites contributing to tumorigenesis, exposure of tissues organs to parasite, and/or parasite-metabolites are particularly susceptible to infection-induced malignancy (Brindley and Loukas, 2017).

2.1. Risk factors leading to bladder cancer and cholangiocarcinoma associated to helminth infections

Carcinogenesis is a complex process in which normal cell growth is modified as a result of the interaction of multiple factors (WHO, 2015). Several models have been proposed to explain the rise of cancer due to opisthorchiasis and UGS (Sithithaworn et al., 2012; Sripa et al., 2012; Honeycutt et al., 2014).

In regions where opisthorchiasis is highly endemic the major risk factors for the development of CCA are either chronic inflammation and persistent parasitism that induce continuous damage of the biliary epithelium (Sripa et al., 2012; Blechacz et al., 2011; Razumilava and Gores, 2013). Nonetheless, several other factors might be involved including increase of reactive oxygen and nitrogen species (ROS/RNS) or interleukin-6 (IL-6) among others factors derived from immune host attack directed against the worms (Jusakul et al., 2011), secretion by the parasite of mitogens such as granulin, thioredoxin and other mediators (Satarug et al., 1998; Sripa et al., 2012), dietary nitrosamines (Sripa et al., 2012), and others (Figure I-7). Additional to these factors, recently, liver fluke derived oxysterol-like metabolites that potentially damage host DNA have been related to carcinogenesis associated with opisthorchiasis (Vale et al., 2013).

As for opisthorchiasis-associated CCA, several models for UGS-associated bladder cancer have been proposed over the years (Figure I-7) (Honeycutt et al., 2014). Several factors including exposure to industrial and agriculture dyes, tobacco smoke, vitamin A deficiency, and exposure to low doses of nitrosamines are considered as potential carcinogenesis initiators in some models (Mostafa et al., 1999; Honeycutt et al., 2014). Recently, experimental studies using non-cancerous Chinese Hamster Ovary (CHO) cell lines exposed to secretions and lysates of *S. haematobium* eggs and adults worms stimulates cellular proliferation, migration, and invasion, inhibition of apoptosis, upregulation expression of Bcl-2 and facilitation of loss of p27 (Botelho et al., 2009, 2011a, b) which are hallmarks of tumorigenesis and cancer cell survival (Hanahan and Weinberg, 2011). Recent studies using human epithelial cell lines (HCV29) co-cultured with *S. haematobium* eggs also demonstrated that the presence of eggs stimulates cell proliferation (Nacif-Pimenta et al., 2019). Moreover, in an animal model, intravesical administration to mice of *S. haematobium* antigens

and eggs induced urothelial dysplasia (Botelho et al., 2011b). This finding suggests that malignization of the bladder caused by *S. haematobium* infection is independent of the presence of nitrosamines (Botelho et al., 2011b). Among to these potential risks, catechol estrogens-like metabolites derived from *S. haematobium* might play an important role as chemical carcinogens in UGS-associated carcinogenesis (Correia da Costa et al., 2014; Brindley et al., 2015). Metabolites of estrogen have long been considered as carcinogenic chemicals and implied in several other cancers like breast, thyroid, prostate, non-Hodgkin lymphoma, among others (Cavalieri et al., 2002 and 2006; Gaikwad et al., 2009; Zahid et al., 2013).

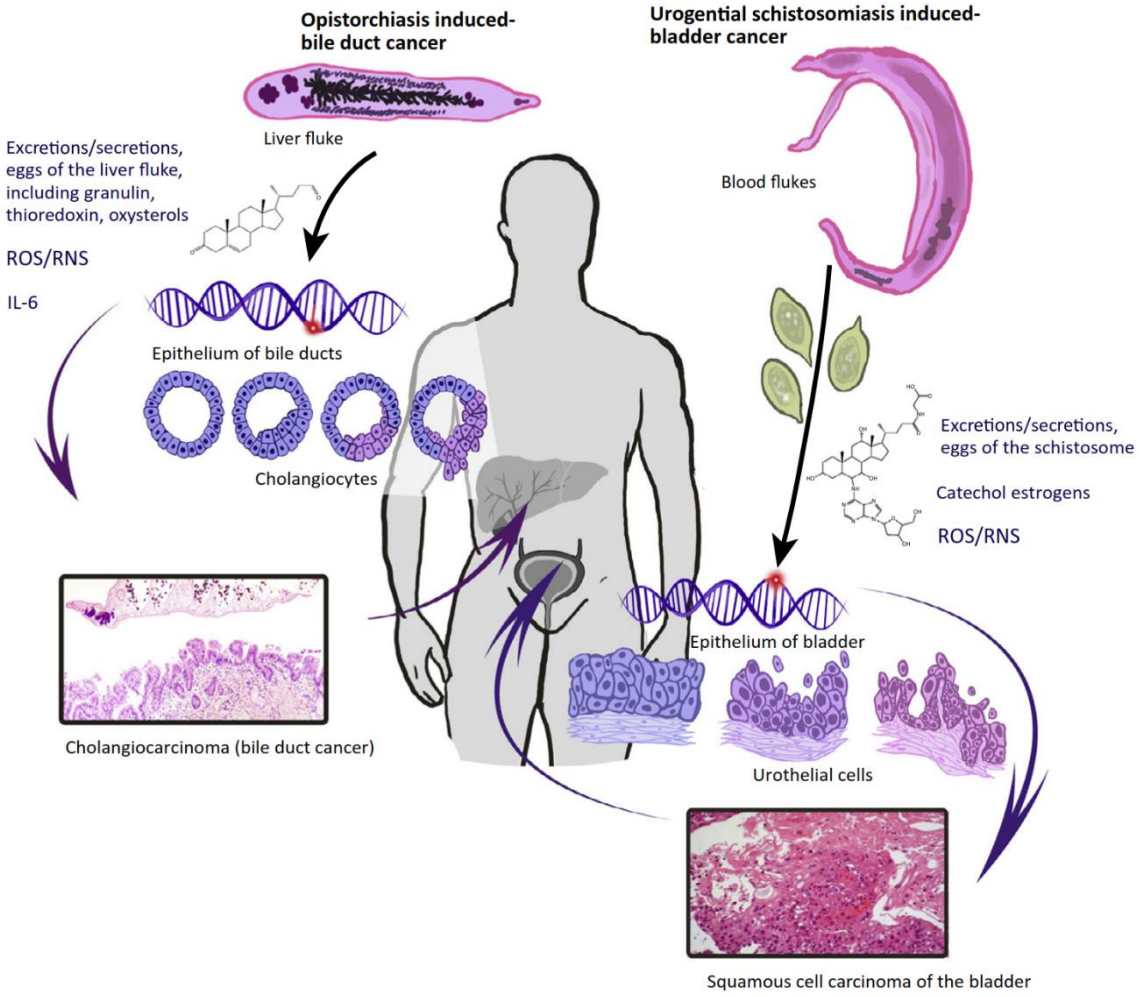

Figure I-7. Schematic representation of multifactorial processes that can contribute to the development of the biliary and bladder/urogenital tract cancers resulting from infection with the liver fluke *O. viverrini* and *S. haematobium*. On the left side of the diagram is depict the adult stage of *O. viverrini* and small bile ducts with cholangiocytes. On the right side of the diagram depicts the adult *S. haematobium*, and the urothelium lining the urinary bladder. Several factors may be related to the development of carcinogenesis associated with opisthorchiasis and UGS (Figure reproduced from Brindley et al., 2015).

Estrogen and oxysterol-like metabolites within parasite origin might have a potential role in helminth infection-associated carcinogenesis. Which evidence associates these metabolites with infection caused by these helminths? In the next subchapter, we will explore the evidence of estrogen and oxysterols-like metabolites associated with UGS and opisthorchiasis.

2.2. Evidences of parasitic catechol estrogens and oxysterol-like metabolites associated to helminth infection

The first association of estrogen/estradiol to UGS occurred during the examination of human cases from Angola that presented elevated levels of estradiol in sera but not luteinizing hormone or testosterone (Botelho et al., 2009). Most likely, the elevation of estradiol observed was not related to human production of the hormone, but it could be attributed to schistosome production of estradiol-related metabolites (Botelho et al., 2009). In fact, using liquid chromatography-mass spectrometry (LC-MS/MS) several estrogen-related metabolites including catechol estrogen quinones and catechol estrogen quinones-DNA adducts have been characterized in sera of UGS patients (Botelho et al., 2010, 2013) as well as in urine (see Section III-chapter 1) and developmental stages of parasites, including eggs (Botelho et al., 2013). The catechol estrogens detected (Botelho et al., 2010, 2013) are similar to those implicated on breast, thyroid and other cancers (Cavalieri et al., 1997, 2002, 2006) with hydroxylation and subsequent oxidation at positions C-2 and C-3 of the aromatic ring (Botelho et al., 2013; Gouveia et al., 2013; Correia da Costa et al., 2014). The pathways and enzymes involved in the production of estrogen-like metabolites by schistosomes were predicted and reviewed by our research group (Vale et al., 2017b, see Section VI-Appendix 1). This study is based on estrogen metabolism that is mediated by diverse cytochrome P450 enzymes (Vale et al., 2017b). The P450 metabolism of estrone and estradiol by 17β-hydroxysteroid dehydrogenase generate 2-hydroxyestrogen and 4-hydroxyestrogen (Cavalieri et al., 1997, 2002). Since schistosome genome encodes an ortholog of estradiol 17β-dehydrogenase that participates in the synthesis pathway of estradiol (Young et al., 2012), it is reasonable to speculate that parasite might exploit the production of estrogen-like metabolites during parasitic developmental stages (Correia da Costa et al., 2014; Vale et al, 2017b). The subsequent oxidation to catechol estrogen quinones might be performed by enzymes of P450 family as CYP1B1, CYP2E1 or CYP1A1 (Cavalieri et al., 1997, 2002). To a deeper understanding of how these metabolites and DNA adducts are formed, during this thesis, we performed *in vitro* studies to evaluate the influence of P450 enzymes in their formation (see Section III-Chapter 3).

The catechol estrogens quinones detected (Botelho et al., 2010, 2013) might react directly with host DNA via Michael addition or indirectly via generation of reactive

oxygen species (ROS) leading to DNA damage either by forming apurinic sites or DNA oxidation (Cavalieri et al., 1997, 2002). As demonstrated in Section III – Chapter 1 of the present thesis, the evidence of DNA damage was detected in the urine of UGS patients. The catechol forms of estrogens can be inactivated through methylation mediated by catechol-*O*-methyltransferase (COMT), conjugation of the catechol estrogen quinones with glutathione-*S*-transferase (GSH), and enzymatic reduction (Cavalieri et al., 2002, 2006, 2011; Zahid et al., 2013). These processes prevent the accumulation of highly reactive metabolites. However, if they are insufficient, catechol estrogen quinones might accumulate and lead to DNA damage (Cavalieri et al., 1997, 2002; Yager and Davidson, 2006; Cavalieri and Rogan, 2016). During UGS the activity of phase I and II drug-metabolizing enzymes in the urothelium of the bladder might be altered which interfere with the detoxification of xenobiotics and other homeostatic processes (Vale et al., 2017b). The GSH activity decreases during infection, and therefore, the highly reactive metabolites derived from parasite might accumulate in the urothelium of the bladder, and potentially increase the formation of DNA adducts. Indeed, DNA forms of catechol estrogen quinones were also detected in urine (Section III-Chapter 1) and sera of UGS patients (Botelho et al., 2010, 2013). Most likely, the formation of DNA adducts is a consequence of the interaction of parasitic metabolites with chromosomal DNA (Botelho et al., 2011, 2013). These evidences suggest that these reactive metabolites of estrogen might act as mutagens in UGS-induced bladder cancer (Correia da Costa et al., 2014; Brindley et al., 2015).

Using the same technique, i.e. LC-MS/MS, estrogen and mostly oxysterols-like metabolites have been characterized in *O. viverrini* adult worms from experimentally infected hamsters. Several of structures are ramified at C-17 which means that presented discrete and variable chains linked to carbon 17 of the steroid ring (Vale et al., 2013). Oxysterols are products of oxidation of cholesterol either by P450 enzymes or non-enzymatic autoxidation processes (Jaworski et al., 2001; Jusakul et al., 2011). Interestingly, a sole member of the cytochrome P450 family of genes is known from the genome of *Opisthorchis* species (Pakharukova et al., 2012, 2015b; Ershov et al., 2019). Presumably, the oxysterol-like metabolites from *O. viverini* might arise from non-enzymatic reactions with oxidative free radical-like oxygen and nitrogen species (Vale et al., 2013), but it is also reasonable to speculate that parasites might use P450 ortholog for production of these metabolites. Like catechol-estrogens quinones,

oxysterols are potentially mutagenic, genotoxic and display pro-oxidative and pro-inflammation activity that promotes and contribute at several stages of carcinogenesis (Kuver et al., 2012; Loilome et al., 2012; Jusakul et al., 2011, 2012). The association between different types of oxysterols and development of several cancers as colon, lung, breast, and bile ducts have been documented (Jaworski et al., 2001; Kloudova et al., 2017). The *O. viverrini*-infection is also commonly associated with elevation of bile acids, including deoxycholic acid (Vale et al., 2013), a potent tumor promoter in cholangiocarcinogenesis (Sirica, 2005). Bile acids constitute a large family of steroids containing a carboxyl group in the side chain, and bile alcohols have similar products in bile acid biosynthesis or as end products (Griffiths and Sjövall, 2010). Among oxysterols, bile acids, bile alcohols, and similar compounds but conjugated at different positions as bile aldehydes and bile sulfates, were also detected and characterized in extracts of *O. viverrini* adult worms (Vale et al., 2013). Probably, the effect of all these individual metabolites can be anticipated to be structure-dependent, and the occurrence of metabolic conversions can lead to the formation of a complex biologically of active or inactive forms (Vale et al., 2013). Opisthorchiids cannot synthesize cholesterol de novo, thus depending on the host to acquire this key nutrient (Young et al., 2014). Nevertheless, the liver flukes have evolved metabolic pathways highly adapted to a lipid-rich diet from bile and/or cholangiocytes (Young et al., 2012; Vale et al., 2013).

A relation between oxysterol or bile acid-like metabolites from *O. viverrini* and CCA has been hypothesized since the early 1990s (Changbumrung et al., 1990). Recent studies performed in hamsters revealed that the oxysterols, cholestan-3β,5α,6β-triol, and 3-keto-cholest-4-ene, occur at elevated levels in the livers with *O. viverrini*-induced cholangiocarcinoma, and induce DNA adduct formation in cholangiocytes *in vitro* (Kuver et al., 2012; Jusakul et al., 2012). As with UGS, these reactive metabolites of cholesterol might be as the mutagens that contribute to liver-fluke infection-induced CCA (Vale et al., 2013).

Although why and how these metabolites are acquired or synthesized by the parasites remain elusive, their formation might be related to the physiology of the worms and/or parasite-host interactions that modulate metabolic pathways of steroid hormones and bile acids (Brindley et al., 2015).

2.3. Oxysterols- and estrogen-like metabolites derived from helminth parasites as initiators of cancer?

Cancer is a complex and heterogeneous set of diseases that involves distinct initial and sequential effects on the target cells: initiation, promotion, and progression (Anderson, 1971). The first involves damage to DNA, leading to mutations in one or more cellular genes that control key regulatory pathways of the somatic cell. An essential feature of promotion is the induction of proliferation in the initiated cells by continuous exposure to agents that are not necessarily carcinogenic (Brindley et al., 2015). The progression results in further mutations from genetic instability during the promotion. Liver fluke-induced CCA and blood fluke-associated bladder cancer proceed through three essential steps, and otherwise display the 'hallmarks of cancer' (Mostafa et al., 1999; Honeycutt et al., 2014; Mesri et al., 2014).

Oxysterols and catechol estrogens occur in both *O. viverrini* and *S. haematobium* (Botelho et al., 2010, 2013; Vale et al., 2013). Given that oxysterols and/or catechol estrogens of trematode origin and/or precursors might induce DNA damage (Figure I.8) and mutate genes in other settings (Cavalieri et al., 2014), these metabolites might be considered as potential initiators of chemical carcinogenesis associated to opisthorchiasis and UGS (Correia da Costa et al., 2014; Brindley et al., 2015).

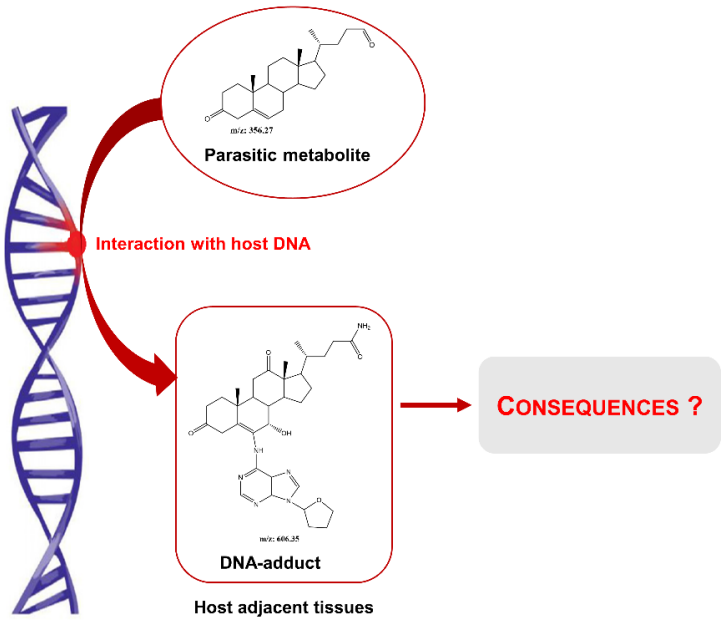

Figure I.8. Metabolites excreted from parasites are highly reactive and might interact with host DNA leading to formation of DNA adducts. Probably this interaction triggers a cascade of events that ultimately culminate with development of infections-associated cancers.

Recently, a potential mechanism was postulated by our research involving helminth derived metabolites (reactive oxysterol and/or catechol estrogen derivatives) and their interaction with host DNA (Correia da Costa et al., 2014; Brindley et al., 2015). Briefly, both types of metabolites within parasitic origin are highly reactive and could react with host DNA yield DNA adducts that consequently induce lesions in chromosomes and promoted host cell DNA damage. The formation of DNA adducts induces continuous DNA oxidation and apurinic sites that eventually escape the DNA repair mechanism leading to mutations. The accumulation of mutations might transform the target cell originating hyperplasia and dysplasia, and also might induce dysregulation of oncogenes and tumor suppressors that ultimately culminate in the development of cancer (Figure I-9) (Correia da Costa et al., 2015; Brindley et al., 2015).

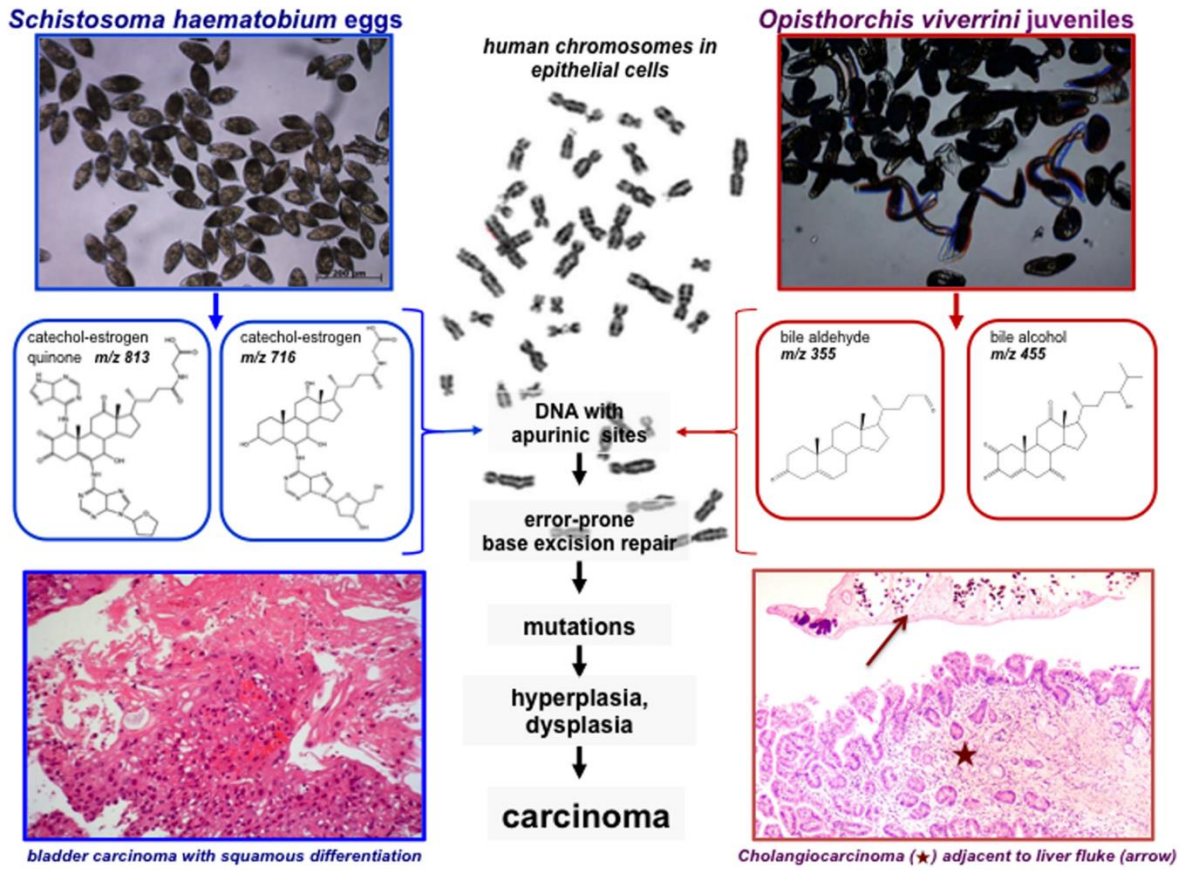

Figure I-9. Postulated of chemical carcinogenesis mediated by oxysterols and estrogen-like metabolites derived from *O. viverrini* and *S. haematobium*. Metabolites derived from *S. haematobium* and *O. viverrini* might interact with chromosomes of target cells inducing DNA damage that eventually is not repaired and leads to the accumulation of mutations. This accumulation might transform the target cell leading to hyperplasia, dysplasia and, ultimately to carcinoma. (Figure reproduced from Correia da Costa et al., 2014)

As mentioned above, the accumulation of mutations could lead to a dysregulation of tumor suppressors. The overexpression and/or alteration of p53 during UGS have been described (Kidane et al., 2014; Santos et al., 2014) and might be related to the production of DNA adducts and oxidation of DNA. The p53 is a tumor suppressor protein involved in diverse pathways as a cellular response to DNA damage, initiation of DNA repair, among others (Habib et al., 2006; Nikoletopoulou et al., 2013). Dysregulation of the expression of the p53 gene may accelerate the tumorigenesis and alter the response of cellular agents that damage DNA (Colley et al., 2014). Studies performed using human epithelial lines (HCV29) co-culture with *S. haematobium* eggs and biopsies from the bladder of UGS patients from Angola demonstrated that p53 is usually altered during *S. haematobium*-infection (Warren et al., 1995; Santos et al., 2014; Nacif-Pimenta et al., 2019). The dysregulation of p53 observed in the urothelium of the bladder associated with *S. haematobium* infection reinforce the notion that UGS alters the cellular process in this tissue and contribute to the development of bladder cancer due to continuous accumulation of mutations (Vale et al., 2017b). Tumor suppressor protein p53 is also a commonly mutated gene in *Opisthorchis viverrini*-related CCA (Jusakul et al., 2015).

The oxysterol/estrogen-DNA adduct mediated pathway postulated (Correia da Costa et al., 2014; Brindley et al., 2015) is in line with the hypothesis recently proposed (Brücher and Jamall, 2014). According to the authors, a sequence of events must occur to originate cancer. First, it is necessary a biological or chemical *stimulus*, followed by chronic inflammation which leads to fibrosis, and a change in the tissue microenvironment. These alterations promote precancerous niche formation which triggers the deployment of the chronic stress escape strategy. If this strategy fails to resolve, the transformation of a normal into a cancer cell occurs originating cancer (Brücher and Jamall, 2014).

3. Current treatment and control for schistosomiasis and opishtorchiasis: a single drug

The morbidity control of opisthorchiasis and schistosomiasis depends entirely on chemotherapy with a single drug, praziquantel (PZQ) (WHO, 2006). The pyrazino-isoquinolone was developed in the 1970s through a collaboration between Merck and Bayern. Initially, PZQ was used for veterinary medicine as a broad-spectrum anthelmintic for companion animals and livestock (Andrews et al., 1983; Groll, 1984). In the late of 1970s and early 1980s, the drug was evaluated in a series of clinical trials against the major human schistosome species (Leopold et al., 1978; Davis and Wegner, 1979; Davis et al., 1979, 1981) followed by open-label clinical trials launched in Asia to assess its efficacy against members of Opisthorchiidae family (Bunnang and Harinasuta, 1981; Rim et al., 1981). For over more than 40 years and until today, PZQ is considered the 'drug of choice' for the treatment of human opisthorchiasis and all forms of schistosomiasis (Cioli and Pica-Mattocia, 2003; Olliaro et al., 2014). Since 2006, PZQ has been extensively used in mass drug administration (MDA) programs in endemic regions as 'preventive chemotherapy' against these helminth diseases (WHO, 2006). Several millions of doses of PZQ have been consumed since implementation of MDA programs and it has been estimated that about 235 million people received PZQ until 2018, only for schistosomiasis (WHO, 2013). Nevertheless, regardless of the efforts of MDA programs to control liver and blood fluke infections, they remain a major public health concern (Andrews et al., 2008; Hughes et al., 2017). According to WHO, the recommended dose of PZQ for opisthorchiasis and schistosomiasis is 40 mg/kg at a single dose in preventive chemotherapy. For opisthorchiasis, PZQ can also be administrated using three doses of 25 mg/kg daily for 2-3 consecutive days for individual treatment (WHO, 1995a). The administration of PZQ in patients with these helminthiases is well tolerated presenting only mild and transient adverse effects including abdominal pain, dizziness, nausea, vomiting, vertigo, headache, rash and hypotension (Haswell-Elkins and Levri, 2003).

The major drawbacks of PZQ, mechanism of action and the potential emergence of resistance were extensively reviewed by Vale et al. (2017a, see Section VI-Appendix II). Briefly, the major shortcomings of PZQ include its inefficacy against juvenile forms including the schistosomula of *Schistosoma* spp. and *Opisthorchis* spp. newly

excysted metacercariae (NEM) (Pakharukova et al., 2015a; Vale et al., 2017a), has poor solubility and an extensive metabolism via hydroxylation of the absorbed drugs to inactive metabolites (Figure I.10) (Olliaro et al., 2014).

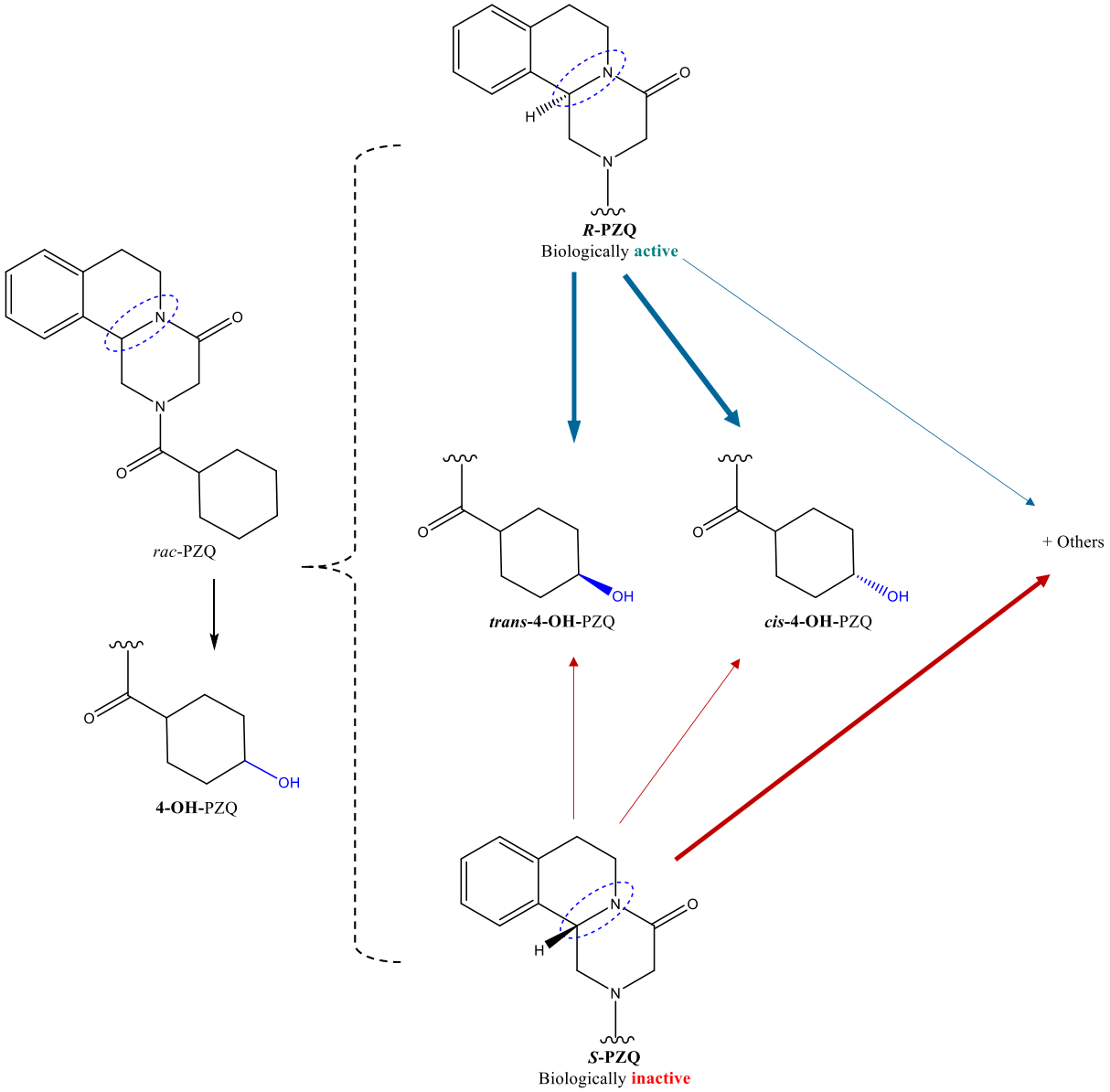

Figure I.10. Metabolism of PZQ mediated by family of CYP450 enzymes. PZQ is a racemic mixture of enantiomers biologically active (*R*-PZQ) and inactive (*S*-PZQ) isomers. The drug is metabolized by CYP450 resulting in 4-OH-PZQ as the main product and other minor enantiomers as *cis*- or *trans*-4-OH-PZQ. Bold green and blue arrows indicate major metabolites of PZQ enantiomers. (Figure adapted from Vale et al., 2017a).

Although administration of PZQ might clear the infection, alone the drug cannot prevent or ameliorate inflammation, fibrosis-related to the infection and re-infection. Therefore, PZQ cannot counteract the risk factor for the development of cancer

associated with these infections (Hughes et al., 2017). Interestingly, some studies in a hamster model of *O. viverrini* infection reported that repeated infection and consequence PZQ re-treatment can increase the risk of CCA due to augment of inflammatory cells which increase oxidative and nitrative stresses and apoptosis (Pinlaor et al., 2004, 2009a). Nonetheless, no conclusive evidence correlates the PZQ-repeated treatment with CCA in human cases (Kamsa-ard et al., 2013).

Regardless of its wide usage, the mechanism of action of PZQ is unknown (also reviewed by Vale et al., 2017a). Nevertheless, it has been postulated that PZQ disrupts ion calcium (Ca^{2+}) homeostasis but how the drug alters it remains unclear (Valle et al., 2003; Greenberg, 2005). The hypothesis that PZQ alters Ca^{2+} channels is supported by finding of studies that employed calcium channels blockers and cytochalasin D (Troiani et al., 2007). PZQ might be a G-protein-coupled receptor ligand, with PZQ acting as an agonist at the human 5-HT_{2B} receptor (Chan et al., 2013). The drug causes immobilization, spasmodic contractions, paralysis of the worm accompanied by tegument damage (Angelluci et al., 2007; Tallina and Redi, 2007), as evidence of extensive swelling, erosin, vacuolisation and peeling (Pakharukova et al., 2015a). The pathological vacuolization of the tegument cells causes leakage of sugars and amino acids and cell lysis leading to the death of parasite (Angelluci et al., 2007; Chan et al., 2013).

Due to reliance of single drug for these two major helminth infections spread worldwide, there is a growing and legitimate concern that resistance to PZQ might evolve (Doenhoff et al., 2008; Merrifield et al., 2016; Trainor-Moss and Mutapi, 2016). Thus far, PZQ resistance is not of clinical concern, however, field and experimental isolates, especially for schistosomes, exhibiting significantly reduced susceptibility or low cure rates have been described, foreboding the emergence of drug resistance in these parasites (Fallon, 1998; Wang et al., 2012). Unlike reported for schistosomiasis, so far, there is no evidence of opisthorchiids resistance to PZQ. Nevertheless, it is plausible to presume that due to its massive and extensive use, the PZQ-resistance and/or decrease susceptibility of liver flukes to the drug will emerge.

Based on these facts, the search for additional or alternative drugs against *Opisthorchis* and *Schistosoma* species has become a public health priority. Curiously, the research for alternative therapies against opisthorchiids has attracted much less

attention than for schistosome. Very little research on this topic is conducted outside of Thailand and Russia.

4. Alternative therapeutic approaches against schistosomiasis and opisthorchiasis

Over the past years, extensive efforts been made through the synthesis of derivates of PZQ and evaluation of anthelmintic activity either *in vitro* or *in vivo* (Doenhoff et al., 2008; Liu et al., 2012). Unfortunately, these derivatives did not present better activity in comparison to the parent drug (Vale et al., 2017a). Therefore, there is a reawakening of the need to search for alternatives to PZQ. Strategies as the development of combinations of PZQ with drugs or other active agents (Keiser et al., 2011) and drug repurposing (Panic et al., 2014; Cowan and Keiser, 2015) might constitute promising and efficient tools against these helminthiases (Panic et al., 2014).

4.1. Drug repurposing and combination of drugs

The concept of drug repurposing is based on the identification of new therapeutic indication for already approved or investigational drugs that are outside of the initial purpose (Ashburn and Thor, 2004). This is a useful strategy that offers several advantages to accelerate the drug development process due to lower cost, reduce risk, and decreased time to availability of preclinical data (Padhy and Gupta, 2011). On the other hand, rational combination therapy consists of the use of one or more drugs or a combination of drugs with other active agents. Initially, the rational combination of chemotherapy was developed for tuberculosis and other bacterial infection (Kerantzas and Jacobs, 2017). Nowadays, its use has been extended for chemotherapy of cancer, acquired immune deficiency syndrome (AIDS) (Maenza and Flexneer, 1998), and for malaria (Nosten and Brasseur, 2002; Wells et al., 2015). The major goals of combination chemotherapy are minimized or delay the potential arises of resistance (Maenza and Flexner, 1998; Wells et al., 2015; Kerantzas and Jacobs, 2017), and/or achieve additive/synergistic effect that could be translated in reduced doses of drugs along with the reduction of adverse effects (Kerantzas and Jacobs, 2017).

The state of the art regarding drug repurposing and combination of drugs, as well the use of antioxidants either alone or combine with PZQ and other drugs against schistosomiasis, were extensively reviewed by Gouveia et al. (2018; see Section VI-Appendix 3). Here presents a brief summary of the studies described in our review.

Several classes of pharmacological agents including anthelmintics, antimalarials, anti-inflammatory, contraceptives agents, have been evaluated and suggested for drug repurposing against schistosomiasis. The drugs including artemisinin´s derivates (e.g. artesunate (AS) and artemether (ART)) and mefloquine (MFQ) which are widely acknowledged for their antimalarial activity (Ashley and White, 2005) have been demonstrated an interesting antischistosomal activity. These compounds are highly active against juvenile's worms (Ashley and White, 2005) whereas PZQ is only effective against adult worms. Thus, antimalarials counteract one of major shortcoming of PZQ. Over the years, antimalarials were tested either alone or combined with PZQ and evaluated not only in the laboratory but also in clinical trials where achieved promising results against schistosomiasis (De Clercq et al., 2000; Borrmann et al., 2001; Mamhoud and Botros, 2005; Hou et al., 2008; Inyang-Etoh et al., 2009; Keiser et al., 2009a; Xiao et al., 2009, 2011; Yunusa et al., 2016). Due to their different mode of action, the combination of artemisinin´s derivatives and PZQ should lead to the elimination of parasites in larval form and adult worm which might be very useful in regions with an elevated rate of re-infections. A recent meta-analysis confirms that antimalarials as artemisinin derivatives used in combination with PZQ exhibited the increased cure rates in schistosomiasis treatment. Also, suggest that repeated doses of artemisinin derivatives might play a prophylactic role due to its activity against the larvae stage of schistosomes (Pérez del Villar et al., 2012). MFQ is considered one of the best antischistosomal drugs being active not only against larvae but also adult schistosomes. This antimalarial drug display better antischistosomal activity in comparison to artemisinin derivatives and PZQ. Additionally, MFQ combine with AS exhibit higher cure rates against schistosomiasis mansoni and UGS (Xiao, 2013). Other pharmacological agents including anti-inflammatory, ibuprofen and naproxen, antifibrotic, lipid lowering agents as atorvastatin, and synthetic lipids, edelfosine, were also being evaluated against schistosomiasis. Although the anti-inflammatory agents do not exhibit antischistosomal activity *per se*, they played a role in amelioration of inflammation, biochemical and histopathological consequences related to intensity of

infection, and decrease in granuloma diameter (Mahmoud et al., 2002). Similarly, antifibrotic agents as β-aminopropionitrile-monofumarate salt and β-aminopropionitrile combined with PZQ reduced sizes of granulomas and alleviate the host resistance to challenge infection. The administration of combine regimens of these drugs with PZQ lead to absence of worm recovery and only dead ova were observed. The results achieved by combine regimens were better than monotherapies alone (Giboda et al., 1992; Hassan et al., 2003). Combine lipid lowering agent and injectable contraceptive medroxyprogesterone acetate result in severe tegumental damage and significantly decrease the total number of *S. haematobium* worms recovered from infected hamsters. Intriguingly, female worms were less susceptible to either drugs alone or combine in comparison to males (Soliman and Ibrahim, 2005). Unlike PZQ, edelfosine, a synthetic lipid, presented antischistosomal activity against larvae and adult stage of *S. mansoni*. Apparently, the antischistosomal activity of this synthetic lipid depend on its action on the tegumental structure (Yepes et al., 2014). In addition, combined regimens of edelfosine plus PZQ *in vivo* not only eliminated the schistosomes developmental stages but also improve histopathological parameters as reduced granuloma size and hepatomegaly. Furthermore, it potentiated anti-inflammatory actions and favor resistance to re-infection render eldefosine a promising candidate for a prophylactic treatment of schistosomiasis (Yepes et al., 2015).

The paucity of alternatives against opisthorchiasis might relate to a certain delay in the studies into the systematic biology of opisthorchiids resulting from a relatively lower abundance of the corresponding helminthiases as compared with schistosomiasis. Consequently, insufficient research attention towards this problem. Similarities between the morphology, anatomic structure and physiological process typical of trematodes suggest that the label extension and drug repurposing can also be successfully applied to the development of opisthorchicidal drugs (Mordvinov and Furman, 2010). A few interesting candidates for drug repurposing and drug combinations against opisthorchiasis have studied over the past years. Below is briefly present the most promising drugs and its effects against either opisthorchiids or pathologies associated with infection.

During the 1980s, the potential opisthorchidal activity of drugs albendazole (ABZ) and mebendazole (MBZ), was evaluated in laboratory studies (e.g. *in vitro* and *in vivo*) and clinical trials. These drugs have been widely and effectively used in the treatment

and control of soil-transmitted nematode infections (Utzinger and Keiser, 2004). The administration of drugs to opisthorchiasis patients twice daily for 3-4 days only achieved moderate cure rates, albeit with egg reduction rates above 90% (Pungpark et al., 1984). Treatment with MBZ was also effective in eliminate opisthorchiasis presenting low toxicity (Jaroonvesama et al., 1981). In a recent clinical trial, administration of ABZ eliminate the *O. viverrini* infection in children co-infected with nematodes, and was more effective than MBZ (Soukhathammavong et al., 2012). Recently, a novel complex of ABZ with polysaccharide arabinogalactan demonstrated to be highly effective against *O. felineus in vitro* presenting an anthelmintic activity at significantly lower doses than ABZ, and also showed lower acute toxicity and hepatotoxicity than the parent compound (Chistyachenko et al., 2015). Since antimalarials shown potent activity against schistosomes, they may also have potential for treatment and control of opisthorchiasis. Semi-synthetic derivatives as artemether (ART) and artesunate (AS) were administered to *O. viverrini*-infected hamsters which result in worm burden reduction between 60-80%. Even at higher dose (600 mg/kg), the complete elimination of the parasite was not achieved and both drugs showed toxic effects (Keiser et al., 2006). In a similar fashion to artemisinin´s, the appealing antischistosomal activity of mefloquine (MFQ) triggered interest in its possible activity against opisthorchiasis. In a similar fashion to observed against schistosomes, MFQ achieved better opisthorchidal activity than AS and ART either *in vitro* and *in vivo*. A single oral dose of MFQ resulted in high worm burden reductions not only against juvenile but also adult *O. viverrini* (Keiser et al., 2009b). The MFQ induces severe tegumental alterations including sloughing, furrowing and bebbling following incubation *in vitro*, suggesting that drug targets the fluke´s tegument (Keiser et al., 2009b). Tribendimine (TBD), was initially developed in an attempt to control tapeworm and threadworm infections in China (Xiao et al., 2005). Laboratory and clinical investigations demonstrated the therapeutic safety of TBD (Xiao et al., 2005). The *in vitro* assays demonstrated that exposure of parasites to TBD at lower drug concentrations lead to its rapid contraction and consequently to death. Administration of a single oral dose of TBD *in vivo* resulted in high worm burden reduction. Similar to MFQ, TBD induces severe tegumental disruption as sloughing, furrowing, and bebbling. Also, TBD damage the oral sucker of parasite leading to a complete closure of the mouth of the liver fluke, however, the *O. viverrini* recovered from infected hamsters remain alive in contrast to observed *in vitro*. Its impressive activity *in vitro* even at lower

concentrations only translated to a moderate worm burden reduction *in vivo* (Keiser et al., 2008). Nevertheless, opisthorchidal activity of TBD was evaluated in two randomised, parallel-group, single-blind, dose-ranging, phase 2 trials in children, adults and adolescent were performed in three *O. viverrini*-endemic villages in southern Laos (Sayasone et al., 2016). Several doses of TBD were evaluated in different ratios according to age of the children. The aim of these trial was to estimate the dose-response relations in terms of cure rate and egg reduction rate. The results obtained demonstrated that TBD has excellent efficacy and tolerability at doses of 100 mg/kg and above. Nonetheless, it should be note it that mainly adults and children presented low-intensity *O. viverrini* infection, thus, further studies including patients with moderate and high intensity are warranted (Sayasone et al., 2016). In a recent clinical trial whose aim was to compare the efficacy of administration of a single oral dose of TBD with two oral doses of PZQ demonstrated that TBD has a slightly lower cure rate than PZQ. Nevertheless, TBD has a similar egg reduction rate to PZQ and leads to fewer adverse events (Sayasone et al., 2018). The opisthorchidical effect of combination of PZQ with TBD was also evaluated either *in vitro* and *in vivo*. While *in vitro* the combination presented a synergistic effect, when both drugs were administered *in vivo* simultaneously or on subsequent days achieved low to moderate worm burden reduction, suggesting antagonistic effects (Keiser et al., 2008). It remains unclear why this apparent contradiction occurs but could be related to pharmacokinetics or pharmacodynamic drug interactions *in vivo* (Sayasone et al., 2016). Through the analysis *in vitro* of anthelmintic activity of various CYP inhibitors against juvenile and adult *O. felineus*, miconazole (MCZ) and clotrimazole (CTZ) were the most effective reducing CYP activity but also decreased the viability of the liver flukes (Mordvinov et al., 2017). The inhibition of CYP activity might be relevant since parasite encodes a CYP enzyme active on parasite tissues that could be essential for its survival (Pakharukova et al., 2015b). The activity of these two agents, both approved by the United States Food and Drug Administration, was comparable to that for PZQ (Mordvinov et al., 2017). The combination of CTZ or MCZ with PZQ also lead to contradictory results. Similar findings to those were seen for TBD (Keiser et al., 2013). The synergistic effect of the PZQ+CTZ and PZQ+MCZ observed *in vitro*, unfortunately were not evident *in vivo* (Mordvinov et al., 2017). The low efficacy of these azoles agents could be attributed to low drug concentration in the hepatobiliary

system where parasites reside. Repetitive dosing at constant time intervals may maintain appropriate drug levels in the hepatobiliary system (Pakharukova et al., 2018).

4.2. Antioxidant biomolecules: a promising alternative?

As mentioned in Chapter 2, it has been postulated that *Opisthorchis* spp. and *S. haematobium* produces/excrete metabolites that might be considered as potential initiators of infection-associated carcinogenesis (Correia da Costa et al., 2014; Brindley et al., 2015). Ideally, the treatment for opisthorchiasis and schistosomiasis not only should target the elimination of the parasites but also ameliorate and prevent pathologies associated to the infections. In our point of view, the novel therapeutic alternative should inhibit the formation of potentially carcinogenic metabolites that ultimately might counteract the initiation of carcinogenesis. It has been demonstrated that antioxidants as resveratrol (Resv) and *N*-acetylcysteine (NAC) can block cancer-initiating process associated to breast and other cancers by counteracting the production of carcinogenic metabolites (Zahid et al., 2008, 2010, 2011). Antioxidants are considered pharmacologically safe agents presenting minimal side effects (Ratman et al., 2006; Sindhi et al., 2013), and might prevent DNA damage (Pinlaor et al., 2009b), stimulate some important antioxidant enzymes that regulate the elimination of reactive electrophilic compounds (Soliman et al., 2008) and present and/or enhance anthelmintic activity (Sayed et al., 2008; Seif-el-Din et al., 2011). Therefore, the biological properties of antioxidants biomolecules either alone or combined with drugs might offer a major contribution to therapeutic against these helminth infections in amelioration of associated pathologies and perhaps, ultimately counteract initiation of carcinogenesis.

Biological and natural agents as antioxidant biomolecules have attracted interest against schistosomiasis and opisthorchiasis. Indeed, the use of antioxidants either alone or combine with different class of drugs have been achieve promising results (reviewed in Gouveia et al., 2018, Section VI-Appendix 3). However, clinical trials to assess the inclusion of antioxidants in therapy against these helminth infections have yet to be launched.

Several antioxidants assessed have shown potential antischistosomal activity either *in vitro* and *in vivo* not only against mature (Rojo-Arreola et al., 2014; Rizk et al.,

2012; Mata-Santos et al., 2010; Eraky et al., 2016) as well as in immature forms of schistosomes (Mantawy et al., 2012; Oliveira et al. 2014; Eraky et al., 2016;). Evidences point out that some antioxidants affect the motor activity of the worms *in vitro* suggesting a possible perturbation/dysfunction of neuromuscular system (Oliveira et al., 2012). The neuromuscular systems are a crucial element for schistosomes since they control not only movement, but also oral and ventral suckers involved with parasites attachment. In addition, they support internal organs including the reproductive, excretory and digestive tracts, and maintenance of the female with the gynecophoral canal of the male (Ribeiro and Patocka, 2013; Patocka et al., 2014). Also, antioxidants also target the parasite tegument (Oliveira et al., 2012; Rizk et al., 2012; Eraky et al., 2016) which is a crucial organ for protection against host responses, nutrient uptake for parasite development and growth (De Paula et al., 2016). Besides the antischistosomal activity, it has been demonstrated that antioxidants are capable to restore the activity of antioxidant liver enzymes (El-Ansary et al., 2007; Rojo-Arreola et al., 2014; Soliman et al., 2017), which is usually accompanied by reduction on granuloma size and number resulting in improvement of liver architecture and functions (Rojo-Arreloa et al., 2014; Kamel et al., 2015; De Paula et al., 2016). Another interesting aspect of antioxidants is that they could modulate and immunomodulatory response, promoting alteration in some cytokines (El-Shennawy et al., 2007; Wan et al., 2017) that might be also helpful to reduced size and number of granulomata. Generally, administration of antioxidants concomitantly with antischistosomal drugs improves not only parasitological but also biochemical parameters (Mahmoud et al., 2002; El-Lakkany et al., 2012; Ebeid et al., 2014). Similar effects of antioxidants have been observed against opisthorchiasis. A few antioxidants agent as melatonin (MEL) (Laothong et al., 2010, 2013; Wongsena et al., 2018), curcumin (Curc) (Pinlaor et al., 2009b, 2010; Charoensuk et al., 2011, 2016), xanthohumol (XTH) (Jamnongkan et al., 2018) and plant extracts (Drab et al., 2005; Wonkchalee et al., 2013) were evaluated and achieved informative results not only in elimination of parasites, but also in remission of infection and carcinogenesis. Antioxidants derived from plant extracts exhibits high anti-*O. felineus in vivo* activity exceeding PZQ efficiency. After treatment no eggs were recovered suggesting that compound might block egg production (Drab et al., 2005). By the other hand, antioxidants as Mel, Curc or XTH diminished the DNA lesions through reduction of oxidative and nitrative DNA damage in the nucleus of the bile duct epithelium, suppress the inflammatory responses (Laothong et al., 2010 and

2013; Pinlaor et al., 2009), reduce status changes including iron accumulation, periductal fibrosis, and even suppress development of CCA in experimentally rodent model of opisthorchiasis viverrini and administration of *N*-dinitrosomethylamine (NDMA) in hamsters. (Wonkchalee et al., 2013; Jamnongkan et al., 2018). These ameliorations increase the survival of animals (Laothong et al., 2010, 2013). Apparently, antioxidants might exert a chemopreventive effect against liver injury and CCA, at least in the hamster model (Laothong et al., 2010, 2013; Wongsena et al., 2018). Antioxidants as XTH may repress CCA development via antioxidant activity through protection of cholangiocytes from oxidative stress (Jamnongkan et al., 2018). Generally, combination of these antioxidants with PZQ improve the histopathological features mentioned above (Pinlaor et al., 2010; Wonkchalee et al., 2013; Charoensuk et al., 2016).

Considering the studies mentioned above, combination among different agents with PZQ or other anthelmintic drugs might be a possibility to overcome limitations of PZQ. Additionally, the administration of antioxidants in rodent models of these helminth infection ameliorate the infection-associated pathologies and enhance antioxidant and immunological responses. Notably, the use of antioxidants followed by treatment with PZQ can lead to suppression of CCA. Studies related to the effect of antioxidant against UGS-associated cancer are scarce. This could be related to the difficulty of 1) maintain life cycle in laboratory and 2) establish a reliable animal model of UGS-associated SCC. The combined treatment might present a dual therapeutic effect and could be related to their different modes of action and/or act on different targets. Thus, strategies as drug repurposing and combination of drugs with antioxidant agents should be pursued.

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Section II

Aims of the thesis

II. AIMS OF THESIS

1. Aims of doctoral thesis

The present thesis focused on two main aims.

1) First, contribute to an understanding of the potential role of metabolites derived from helminths as *S. haematobium* and *Opisthorchis* spp. in the infection-associated carcinogenesis. The specific aims within this topic were:

a) provide insights into carcinogenesis of UGS-induced bladder cancer.

Are metabolites present urine of UGS-patients similar to those observed in serum from UGS patients? Is there evidence of detectable DNA in the urine of these patients? If these metabolites promote DNA damage, we should be able to detect molecular signs of chromosomal lesions, as DNA-adducts and the presence of 8-oxodG (Section III-Chapter 1);

b) Understand why infection with only a few helminths induce cancer.

By contrast to *O. viverrini*, its closely relative *O. felineus* is not considered carcinogenic (IARC, 2012). Some evidence suggests that this classification is due to the lack of epidemiological studies (Pakharukova and Mordvinov, 2016). Thus, we performed some biochemical and histopathological studies using hamsters experimentally infected to assess *O. felineus* carcinogenic potential. (Section III-Chapter 2). Does *O. felineus* also produce/excrete metabolites similar to those detected in *O. viverrini*? Is there evidence of interaction of these metabolites with host DNA? Does *O. felineus* infection induce cancer?;

c) comprehend how these metabolites derived from parasites could be formed.

Will the estrogen metabolism associated enzymes (P450 family) be important for its formation? Or can they be formed through non-enzymatic processes? (Section III-Chapter 3).

2) The second principal aim was to develop a novel therapeutic approach that not only targets the parasite but also could improve pathologies associated with infection and ultimately prevent carcinogenesis. As mentioned in the previous section, control and

treatment for these helminth diseases rely on a single drug, PZQ, that targets the parasite rather than the pathological conditions caused by its infection including cancer (Vale et al., 2017). Considering that metabolites produced/excreted by parasites play an important role in the development of cancer associated with infection, it is important to develop a new therapeutic strategy that potentially counteracts their formation. Thus, based on the concepts of drug replacement and/or combination, we propose a novel therapeutic strategy which consists of the combination of different classes of drugs (anthelmintic, antimalarial and anticancer) with antioxidant biomolecules that might present dual mode of action (Figure II-1).

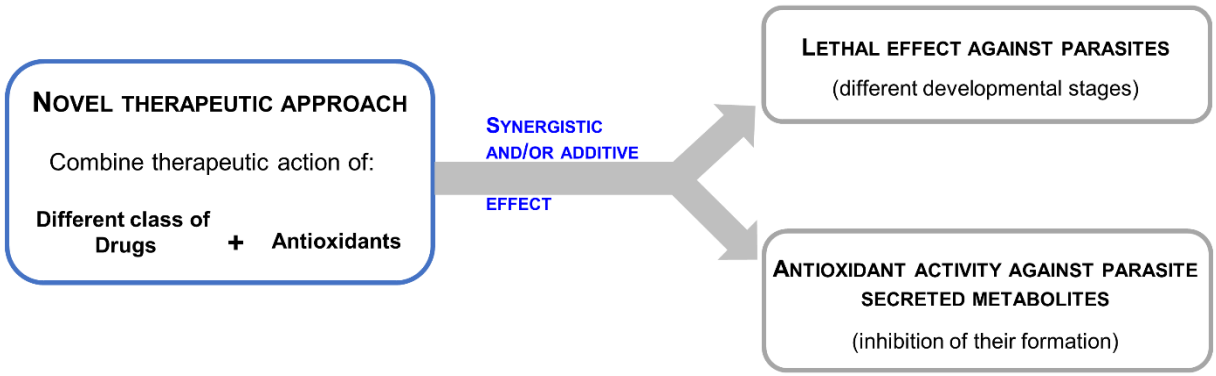

Figure II-1. Novel therapeutic approach and desire effects against opisthorchiasis and schistosomiasis.

Regarding the development of the novel therapeutic strategy, the specific aims were to evaluate its effect on i) elimination of developmental stages, i.e. larva (Section III-Chapters 4 and 5) and adult worms (Section III-Chapter 6) of *S. mansoni*. Due to difficulty of *S. haematobium* grow in rodent and therefore is challenging to maintain its life cycle in the laboratory (Keiser, 2010) the evaluation of novel therapeutic approach was performed in *S. mansoni* model which life cycle is well established in *Centro de Saúde Pública Dr. Gonçalves Ferreira-Instituto Nacional de Saúde Dr. Ricardo Jorge-Porto (INSA-Porto) Laboratories*. Are antioxidants capable of enhancing the activity of drugs? Do antioxidants themselves exhibit anthelmintic activity?; ii) and inhibiting the formation of parasite-derived metabolites. Can the novel therapeutic strategy inhibit the formation of these potentially carcinogenic metabolites? (Section III-Chapter 7).

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Section III

Results

Chapter 1

Estrogen-like metabolites and DNA-adducts in urogenital schistosomiasis-associated bladder cancer

CHAPTER 1. Estrogen-like metabolites and DNA-adducts in urogenital schistosomiasis-associated bladder cancer.

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The paper presented in this Chapter had the main aim provide an understanding of carcinogenesis associated with *Schistosoma haematobium* infection. Based on results obtained in cell lines and animal model, previously, our research group postulated an estrogen-DNA adduct mediated pathway that may be involved in pathogenesis of bladder cancer associated with *Schistosoma haematobium* infection. During this work we search for scientific evidence in humans that supports our postulate which attributes a high relevance role to estrogen metabolites in the molecular mechanisms implicated in UGS-associated carcinogenesis. For that purpose, biological samples (urine and bladder mucosa tissue) were collected from a group of patients with UGS from Angola, properly characterized from the clinical, histopathological and epidemiological point of view. Urine samples were analyzed using the Liquid Chromatography coupled to Mass Spectrometry (LC-MS/MS). The results demonstrated notable differences among the groups of participants. Furthermore, it demonstrated the presence of specific estrogen metabolites excreted in the urine of patients with UGS but not identified in a urinary metabolite database of healthy humans. These metabolites were identified as catechol estrogen quinones (CEQ) and CEQ-DNA adducts (molecules resulting from the interaction of CEQ with host DNA) which some of them had been identified previously on developmental stages of parasite. Additionally, novel metabolites derived directly from 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), an indicator molecule for DNA damage, were identified in urine of all UGS cases. These metabolites can be expected to provide deeper insights into the carcinogenesis UGS-induced bladder cancer, and as biomarkers for diagnosis and/or prognosis of this neglected tropical disease-linked cancer.

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Estrogen-like metabolites and DNA-adducts in urogenital schistosomiasis-associated bladder cancer

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ABSTRACT

An estrogen-DNA adduct mediated pathway may be involved in the pathogenesis of the squamous cell carcinoma of the bladder associated with infection with the blood fluke *Schistosoma haematobium*. Extracts from developmental stages of *S. haematobium*, including eggs, induce tumor-like phenotypes in cultured cells. In addition, estrogen-derived, reactive metabolites occur in this pathogen and in sera of infected persons. Liquid chromatography-mass spectrometry analysis was performed on urine from 40 Angolans diagnosed with urogenital schistosomiasis (UGS), half of who also presented UGS-associated squamous cell carcinoma and/or urothelial cell carcinoma. The analysis revealed numerous estrogen-like metabolites, including seven specifically identified in UGS cases, but not reported in the database of metabolites in urine of healthy humans. These schistosome infection-associated metabolites included catechol estrogen quinones (CEQ) and CEQ-DNA-adducts, two of which had been identified previously in *S. haematobium*. In addition, novel metabolites derived directly from 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG) were identified in urine of all 40 cases of UGS. These metabolites can be expected to provide deeper insights into the carcinogenesis UGS-induced bladder cancer, and as biomarkers for diagnosis and/or prognosis of this neglected tropical disease-linked cancer.

Introduction

Schistosomiasis is one of the major neglected tropical diseases and it is considered the most important helminthic disease of humanity in terms of morbidity and mortality. More than 90% of the cases occur in Africa, of which about two-thirds are caused by *Schistosoma haematobium* [1–5]. Indeed, the number of cases of infection with *S. haematobium* may far exceed that previously predicted so that urogenital schistosomiasis (UGS) may represent the most common infection or even adverse health condition in sub-Saharan Africa [6]. In addition, female genital schistosomiasis increases the risk of transmission of HIV [7–9], and a recent outbreak in Corsica confirms its re-emergence in Europe [10,11]. Many cases of UGS result ostensibly in only mild symptoms and disease such as hematuria, dysuria, anemia and inflammation of the genital-urinary tract [6,12–16]. However, between 25 and 50% of the UGS cases experience moderate to severe morbidity [6], including renal dysfunction, obstruction of the ureters, and squamous cell carcinoma of the urinary bladder [17–20]. Bladder cancer is a frequent and dire complication of chronic UGS. Case reports indicate that patients with schistosomiasis may develop bladder cancer earlier than uninfected people. The severity and frequency of the sequelae of UGS and of its complications are related to the intensity and duration of the infection [21–23]. Moreover, infection with *S. haematobium* is classified as a Group 1 biological carcinogen by the World Health Organization (WHO)'s International Agency for Research on Cancer (WHO IARC) [24] although the cellular and/or molecular mechanisms linking *S. haematobium* infection with carcinogenesis have yet to be defined [25,26]. It has been known for several decades that bladder cancer, especially squamous cell carcinoma (SCC), a distinctly malignant, poorly differentiated neuroendocrine neoplasm [16], was geographically associated with UGS, i.e. areas endemic for schistosomiasis haematobia [20,24,27]. In regions with high worm burdens, and a high risk of exposure to

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S. haematobium infection, SCC is the most frequent histological type, whereas urothelial cell carcinoma (UCC) is predominant in non-endemic locations [22–24,26,28,29].

An estrogen-DNA adduct mediated pathway in *S. haematobium*-infection associated bladder cancer has been postulated [27,30,31]. We have identified and characterized by liquid chromatography-mass spectrometry (LC-MS/MS) novel estrogen-like metabolites, present in the lysates and secretions of *S. haematobium* worms and eggs [27], and in sera of UGS cases [30]. Moreover, lysates of *S. haematobium* induce tumor-like phenotypes; Chinese Hamster Ovary cells exposed *in vitro* to the lysates exhibit marked proliferation and induce sarcoma formation when inoculated into nude mice. Additionally, the cells display increased duration of S phase, decreased apoptosis, down-regulation of the p27 tumor suppressor, and up-regulation of anti-apoptotic protein Bcl-2 [25,27,30,31]. Eggs of *S. haematobium* stimulate cellular proliferation, interfere with apoptosis, increase oxidative stress, and induce a genotoxicity on HCV-29 cells, derived from human urothelial cells [27,30–33]. In addition, 8-nitroguanine forms via inducible expression of nitric oxide synthase in Oct3/4-positive stem cells in UGS-associated bladder cancer tissue [34], and DNA nitrative and oxidative mutations characterized by 8-nitroguanine and 8-hydroxy-2′-deoxyguanosine (8-oxodG), have been implicated in the promotion of inflammation-mediated carcinogenesis by infection with *S. haematobium* [35].

Here we undertook analysis of urine from persons with urogenital schistosomiasis (UGS) living in endemic areas in Angola. Liquid chromatography diode array detection electron spray ionization mass spectrometry (LC-ESI-MS) revealed the presence of numerous estrogen metabolites in the urine of the study participants. Seven of these molecules were specifically identified in urine of the UGS cases but, notably, were not described in the recently reported metabolome of urine from healthy humans [36]. These metabolites were potentially reactive with host DNA: the molecules were either catechol estrogen quinone (CEQ) derivatives or CEQ-DNA-adducts. In addition, novel molecules derived from 8-oxodG during UGS were also identified.

Material and methods

Ethics statement

Informed consent was obtained directly from all participants or parents of underaged participants. Clinical-pathological information was obtained from clinical records of the participants. The Ethics Committee of Agostinho Neto University, Luanda, Angola, approved all procedures employed in this investigation.

Participants assigned to three categories

The participants were assigned to three groups: Group I included cases with urogenital schistosomiasis (UGS) and squamous cell carcinoma of the bladder (SCC); Group II included cases with UGS and with urothelial cell carcinoma (UCC), both histological types SCC and UCC (SSC+UCC) and papillary urothelial neoplasm of low malignant potential (PUNLMP); and Group III included cases with UGS but without cancer. Specifically, Groups I and II included discrete types of bladder cancer: Group I, seven participants with SCC; Group II included 11 cases of urothelial bladder carcinoma, essentially urothelial cell carcinoma (UCC) or mixed with SCC (Tables 1 and 2). Group III included 22 participants presenting non-neoplastic lesions such as chronic inflammation at various grades, including dysplasia, hyperplasia and metaplasia. Table 2 summarizes these details and Supplementary Table S1 provides clinical information and laboratory findings for all 40 participants.

Clinical cases, urine samples

Urine from 40 Angolans (67.5% females, 32.5% males), patients at the Sagrada Esperança Clinic and Hospital Américo Boavida, Luanda, was studied. Cases of urogenital schistosomiasis (UGS) were diagnosed when urine or tissue samples were positive for *S. haematobium* eggs by cystoscopy and/or biopsy. The median age of the UGS cases was 33.5 years, range 12 to 82 years, as described [37]. The study participants who donated the urine resided in the suburban regions of Luanda but most were originally from other Angolan provinces known to be endemic for infection with *S. haematobium* (http://www.who.int/schistosomiasis/epidemiology/en/angola_namibia.pdf). The detection of eggs of *S. haematobium* was undertaken by filtering 10 ml urine through a polycarbonate filter with a pore size of 12 μm in diameter (Millipore, UK). The slides were examined at ×100 under a compound microscope, according to guidelines provided by the WHO, http://www.who.int/mediacentre/factsheets/fs115/en/. One mg/ml final concentration of ascorbic acid was added [38–40] to a matched 10 ml sample, after which urine was stored at –20 °C, and freighted cold to Porto, Portugal for further analysis (below).

All the participants were examined by ultrasonography. Ultrasonographic studies of these participants with UGS revealed irregularities of the inner surface of the bladder wall and, in some cases, localized thickening of bladder wall protruding into the lumen was evident (not shown). The participants underwent cystoscopy and biopsy of the mass and corresponding adjacent mucosa. The apparently normal urothelium of cases without noticeable tumor mass were also subjected to random biopsy. All biopsies of apparently normal urothelium and tumor-adjacent mucosa presented benign/pre-malignant lesions, i.e. papilloma (P), chronic inflammation, urothelial hyperplasia, epidermoid metaplasia or dysplasia. Malignant lesions included papillary urothelial neoplasm of low malignant potential (PUNLMP), urothelial (= transitional) cell carcinoma (UCC), squamous cell carcinoma (SCC), and/or both UCC and SCC, as summarized in Table 1. Biopsy samples were examined and scored by co-author CL, with the pathological diagnosis based on the 2004 grading criteria of the WHO [41]. These criteria were used to organize the participants into the three groups summarized in Tables 1 and 2.

Table 1
Brief epidemiological parameters of study participants in Angola.

Parameter/characteristic	Number	Percentage
Number of participants	40	
UGS	40	100
UGS and associated cancers	18	45.0
UGS participants (Group I, II, III)	40	100
Age in years (mean)	33.5	
Range	12–82	
Gender		
Female	27	67.5
Male	13	42.5
Benign lesion		
Urothelial hyperplasia	12	30.0
Chronic inflammation	12	30.0
Epidermoid metaplasia	12	30.0
Dysplasia	4	10.0
Papilloma	1	2.0
Malignant lesion		
SCC	7	17.5
UCC	5	12.5
SCC + UCC	4	10.0
PUNLMP	2	5.0

UGS, urogenital schistosomiasis; SCC, squamous cell carcinoma; UCC, urothelial cell carcinoma (= transitional cell carcinoma); PUNLMP, papillary urothelial neoplasm of low grade malignant potential.

Liquid chromatography diode array detection electron spray ionization mass spectrometry

Samples were prepared and processed using liquid chromatography diode array detection electron spray ionization mass spectrometry, as described in References 27 and 30. In brief, methanol was added to the urine samples to 50% (volume/volume) and the samples stored at 4 °C until needed. Methanol displays acceptable

Table 2
Numbers and participant codes of samples of bladder tissue in the three study groups of persons with urogenital schistosomiasis (UGS) and with or without bladder cancer. The groups were assigned according to epidemiological and histological criteria. Code names were employed to de-identify the participants.

Group*	Disease status	Participant code name
I	SCC	B12, B20, B22, B27, B35, B37, B44
II	UCC	B2, B3, B6, B30, B42,
	UCC + SCC	B1, B5, B31, B38,
	PUNLMP	B14, B23
III	*Infection with S. haematobium*; with granuloma inflammation; Without bladder cancer (endemic or non-endemic)	B4, B8, B9, B10, B11, B13, B15, B16, B17, B18, B19, B21, B25, B26, B28, B29, B32, B33, B34, B36, B39, B43

SCC, squamous cell carcinoma; UCC, urothelial cell carcinoma (= transitional cell carcinoma), PUNLMP, papillary urothelial neoplasm of low malignant potential.
* All participants in the three groups, Groups I, II and III, were UGS positive.

chromatographic performance in terms of separation and sensitivity, with short gradient times [42]. High performance liquid chromatography (HPLC) coupled with mass spectrometer (MS) was employed to investigate relevant molecular species excreted in the urine. The MS analysis was performed within a LTQ Orbitrap XL mass spectrometer (Thermo Fischer Scientific, Bremen, Germany), fitted with an ultraviolet (UV) photo diode assay (PDA) detector. Analysis of samples involved a single injection of 20 µl of urine for chromatographic separation with a Macherey-Nagel Nucleosil C_{18}-column (250 mm × 4 mm i.d.; 5 µm particle diameter, end-capped). The mobile phase consisted of 1% formic acid in water (A)/acetonitrile (B) mixtures. Elution proceeded at a flow rate of 0.3 ml/min. Eluates were monitored for 75 min, run with a mobile phase gradient of 0–5 min, 100% A; 5–10 min, linear gradient from 100% to 80% A; 10–15 min 80% A; 15–50 min, linear gradient from 80 to 40% A; 50–65 min, 40% A; 65–75 min, linear gradient from 40% A to 100% B. Washing the column for 15 min with acetonitrile between each sample minimized carry-over, and also stabilized the column. Data were collected in negative electrospray ionization (ESI) negative mode scanning with an m/z range of 50 to 2000. The capillary voltage of the electrospray ionization was 28 kV, capillary temperature was 310 °C, flow rates of the sheath gas and auxiliary gas (N_2) were set to 40 and 10 (arbitrary units as provided by the software settings), respectively, and gas temperature was 275 °C. At the outset, the workflow was undertaken using three samples in order to confirm the analytical method and to stabilize the column. Thereafter, samples were analyzed in batches of six. Batch effects were not apparent.

Results

Urine estrogen-derived metabolites specifically related to squamous cell carcinoma of the bladder induced by urogenital schistosomiasis

Photo diode array (PDA) detector chromatograms of the UV spectra were obtained from urine. Mass spectral analysis was undertaken in negative mode, i.e. species were detected as [M-H] ions of the main constituents of urine from Groups I, II and III. The PDA chromatograms for each of the 40 urines are presented in Supplementary Fig. S1. Notable differences among the groups of participants were apparent. Seven specific peaks occurred consistently in urine of all participants with UGS (i.e. infected with *S. haematobium*): peaks at retention intervals of 22, 24, 30, 34, 36, 43 and 49 minutes. The m/z and molecules corresponding to these seven peaks are represented in Fig. 1. These peaks are highlighted in yellow (Group I, II) and orange (Group III) on representative PDAs from the three groups shown in Fig. 2.

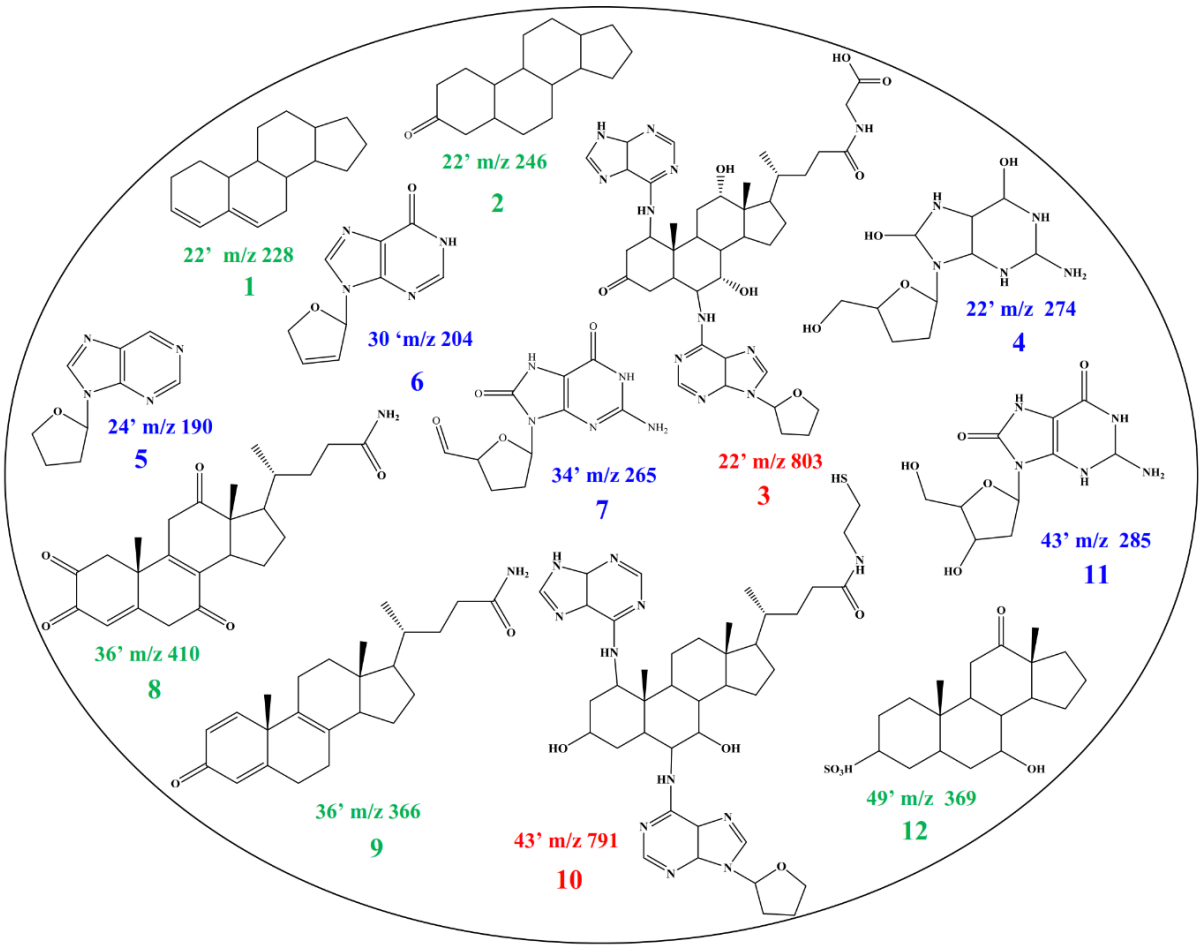

Fig. 1. Representative molecules identified in urine of individuals in Groups I, II and III, but which are not present in the database of the human urine metabolome. Comparing the m/z against a database of the human urine metabolome from healthy individuals [36], these molecules were not identified, indicative that they are related to *S. haematobium* infection. The retention time is the same for the three groups reported. The molecules highlighted by green colored font correspond to derivatives of catechol estrogen derivatives, red to catechol estrogen quinones-DNA adducts, and blue to derivatives of 8-oxodG.

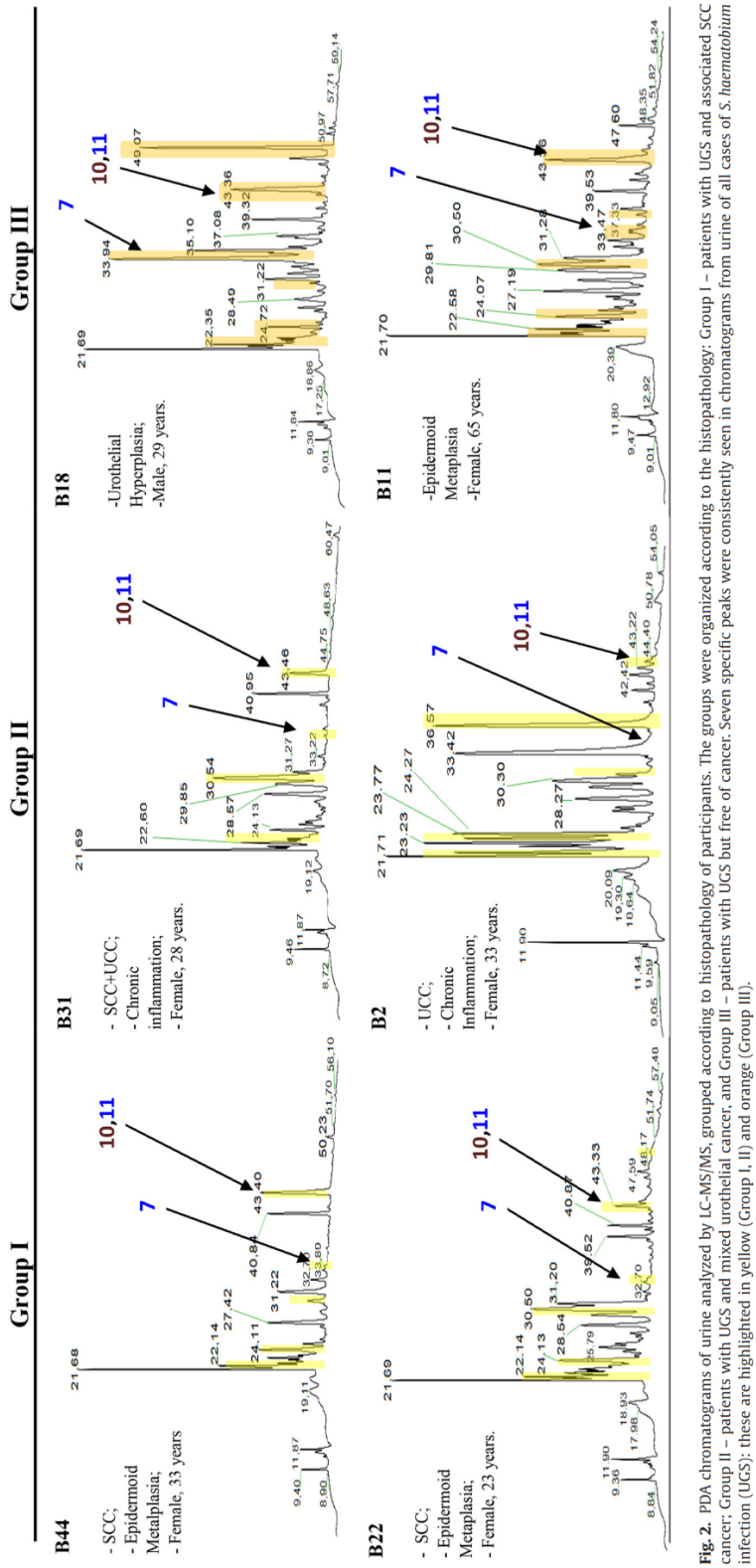

Fig. 2. PDA chromatograms of urine analyzed by LC-MS/MS, grouped according to histopathology of participants. The groups were organized according to the histopathology: Group I – patients with UGS and associated SCC cancer; Group II – patients with UGS and mixed urothelial cancer, and Group III – patients with UGS but free of cancer. Seven specific peaks were consistently seen in chromatograms from urine of all cases of *S. haematobium* infection (UGS): these are highlighted in yellow (Group I, II) and orange (Group III).

There were clear differences between Group III and Groups I/II, illustrated by fewer peaks in urine from participants with UGS-associated bladder cancer (Groups I/II). The peaks with retention times of 22, 24, 34, 36, 43, 49 minutes represented potential biomarkers for the presence of UGS (at some point in time) in individuals with bladder cancer. Further, the peaks at retention times of 34 and 43 minutes may be indicators that infection with S. *haematobium* continued in the persons during the appearance of carcinoma of the bladder. Curiously, the 8-oxodG derivatives related to these peaks, **7** and **11** were more pronounced in participants exhibiting UGS but without cancer, as well as estrogen-DNA-adduct **10** (Fig. 2). This might suggest that DNA oxidation processes are more pronounced in individuals close to the inception of carcinoma (Group III) compared to beyond the appearance of cancer (Group I/II). Once carcinoma in situ manifests, molecules **7**, **10** and **11** (Figs 1, 2) might be useful as urine biomarkers for detection and/or progression of bladder cancer.

The components associated with the indicated peaks were estrogen-like metabolites: derivatives of catechol-estrogen quinones (CEQ) (**1**, **2**, **8**, **9** and **12**) and estrogen quinone-DNA-adducts (CE-DNA-adducts) (**3**, **10**). Other relevant urine metabolites were directly related with 8-oxodG (**4**, **5**, **6**, **7** and **11**). The MS spectra, fragmentation patterns and corresponding retention times for these 12 compounds are presented in Supplementary Fig. S2. All peaks detected in the urine samples were included in the database presented as Supplementary Table S2. The CEQ derivatives are represented with m/z such as 228, 246, 366, 369, 410 and CEQ-DNA adducts present m/z values of 791 and 803 (Fig. 1). CEQ-DNA adducts **3** and **10** have been described previously in eggs and other developmental stages of *S. haematobium* [27,31]. By contrast, these molecules were not present in the catalog of metabolites (metabolome) of urine from healthy people [36].

Metabolites of 8-oxodG are associated with squamous cell carcinoma of the bladder induced by urogenital schistosomiasis

Of the seven peaks discussed above, and highlighted in Fig. 2, retention times of 22, 24, 30, 34, 36, 43 and 49 minutes, several appeared to be related to components that derive directly from 8-oxodG; these exhibited m/z 190, 204, 265, 274 and 285 (**5**, **6**, **7**, **4**, **11**) (Fig. 1). Since 8-oxodG is a representative marker for DNA oxidative damage during oxidative stress [34,43], the detection of 8-oxodG in urine samples herein may be clear evidence that DNA damage occurs in UGS. These reactive oxygen species induce oxidation, nitration, halogenations, and deamination of biomolecules, including nucleic acids, with the formation of toxic and mutagenic products [44]. These, in turn, can lead to DNA damage, eventually inducing mutations that have been implicated in the initiation and/or promotion of inflammation-mediated carcinogenesis [35]. Indeed, we postulate below that the 8-oxodG derivatives, **7** and **11** (retention intervals/peaks at 34 and 43 min, respectively; MS fragmentation profiles presented in Supplementary Fig. S3) represent putative biomarkers for diagnosis and/or prognosis of bladder cancer.

Discussion

Cancer of the urinary bladder is a major complication associated with chronic infection with *Schistosoma haematobium* in Africa and the Middle East [22,24,45,46]. Genetic alterations, chromosomal aberrations, and cytological changes have been described in carcinomas associated with UGS [22,24,47]. *N*-nitroso compounds are implicated as candidate etiologic agents in the process of bladder carcinogenesis [48]. Elevated levels of DNA alkylation damage in carcinomas associated with UGS and a high frequency of G to A transitions in the H-ras gene and in the CpG sequences of the p53 tumor suppressor gene also have been reported [22,47]. These outcomes indicate that UGS-associated SCC arises through a progressive accumulation of genetic changes in epithelial cells. Moreover, there is increasing evidence that endogenous DNA damage is a major etiological factor in human cancers [47].

Indicators of oxidative stress are readily detected in soluble lysates of the adult developmental stages of *S. haematobium* [27], and this mechanism is likely involved in induction of SCC during UGS [47]. Positive correlation between UGS and increase of levels of oxidative stress accompanied by continuous DNA damage and repair in urothelial carcinomas has been observed repeatedly [27,35,44]. In addition, the present findings revealed the presence in urine of molecules **3** and **10** (Fig. 1) known from adult worms and eggs of *S. haematobium* [27]. We have previously implicated a role of these reactive metabolites of schistosome origin in the carcinogenesis of UGS-associated SCC [27]. With respect to interactions of estrogen-related molecules from schistosomes on the endocrine and immune systems of the host, metabolites of estrogens can be considered as carcinogenic chemicals [49,50]. Hydroxylation of estrogens forms the 2- and 4-catechol estrogens involved in further oxidation to semiquinones and quinones, including the formation of the catechol estrogen-3, 4-quinones, the major carcinogenic metabolites of estrogen. These electrophilic compounds react with macromolecules including DNA to form the depurinating adducts that eventually lead to mutation and cancer. Several mechanisms explain the role of estrogen in disease. The better-known hypothesis is that estrogen receptor mediates cell proliferation, increasing errors in DNA replication [51,52]. Another interpretation postulates that estrogen metabolites react covalently with DNA bases by redox cycling or by forming a basic site. Subsequent error-prone repair of the modified DNA generates oncogenic mutations that initiate cancer. The two mechanisms may act in concert [53].

Likewise, metabolism of estrogens and the production of depurinating estrogen adducts can be postulated in a pathway underlying schistosomal-promoted damage to host genes. The carcinogenic effect of this estrogen-DNA adduct mediated pathway could partially explain the link between UGS and SCC of the bladder. UGS induced SCC is clearly multifactorial, however, in like fashion to other infection- and inflammation-related related cancers at large [23–27]. The chromatograms of urine of the UGS cases exhibited few metabolites with high mass range, which we speculate results from chemical processes, including hydrolysis occurring in the aqueous system. This might also explain the presence of more metabolites with higher mass, i.e. m/z 716, m/z 817 in schistosomes themselves. In any event, these findings supported the notion of a carcinogenic effect of an estrogen-DNA adducts mediated pathway in UGS-associated bladder cancer. Globally, the PDA chromatograms included in Groups I and II revealed fewer peaks compared with Group III. These findings are intriguing, and prompt several questions: could this situation reflect a decrease of the excreted metabolites; and might this phenomenon be predictive of cancer progression?

Comparing PDA chromatograms from individuals with UGS and with UGS-associated bladder cancer, there were common peaks that may indicate that the *S. haematobium* infection was responsible for the progression from infection-induced inflammation to bladder cancer. (The structural interpretation of MS data postulated for the novel molecules supported by MS fragmentation analysis is shown in Supplementary Figs S1 and S3.) Moreover, based on comparison of the m/z related to these peaks with a database of the human urine metabolome of healthy persons (from 22 persons in Canada) [36], we conclude that these novel molecules (Fig. 1) resulted from UGS. The peaks at 34 and 43 min retention time are probably indicators that infection with *S. haematobium* continued in the individuals with bladder cancer. The molecules related to peaks, **7**, **10** and **11**, might be useful as biomarkers to detect bladder cancer. These novel molecules proposed are derived directly from

8-hydroxy-2′-deoxyguanosine (8-oxodG), (**7** and **11**), as confirmed by MS fragmentation (Supplementary Fig. S3) or as adduct formation with estrogen (**10**).

8-oxodG is a major chromosomal lesion caused by oxidation, leading predominantly to G to T transversions [34,45–48,54,55]. The formation of 8-oxodG is regulated by local antioxidant capacity and DNA repair enzyme activity; 8-oxodG arises from the spontaneous oxidation of guanine, and is considered the major product of oxidation in DNA and the major source for mutations [33,37,43]. Marked elevation of 8-oxodG level squamous cell carcinoma-derived cells and bladder carcinomas associated with UGS when compared with non-schistosomal carcinomas has been described [47], suggestive of a strong correlation between UGS and increased levels of oxidative stress accompanied by a continuous DNA damage and repair in carcinomas of the urinary bladder [47]. 8-Nitroguanine is produced in the neoplasms of *S. haematobium*-associated bladder cancer patients [35]. Formation of 8-nitroguanine and 8-oxodG was significantly higher in bladder cancer and cystitis tissues than in healthy tissues [35]. 8-oxo-dG is a pivotal marker for measuring the effect of endogenous oxidative damage to DNA and has a factor of initiation and promotion of carcinogenesis [56,57]. Here we confirmed the presence of 8-oxodG in the urine of persons with UGS with or without bladder cancer, which supports the notion that metabolites of estrogen originating from the schistosome induce lesions in host cell chromosomes [31,41].

Whereas mass spectroscopy in isolation cannot assign structure conclusively, and moreover, we have been cautious in over-interpreting the LC-MS data, here we have determined structures that were consistent with the literature [39,49] and with our previous findings related to UGS and tissues and secretions of the parasite [25,27,30–33,37]. Furthermore, the structural interpretation of mass spectrometric data for the novel molecules was supported by MS fragmentations (Supplementary Figs S2, S3). In overview, these metabolites were excreted in urine and, based on molecular structure they may originate in the schistosome. We have previously reported metabolites **3** and **10** in *S. haematobium* eggs and adult worms [27]. Each of the metabolites shown in Fig. 1 potentially can react with DNA to induce apurinic sites, leading to DNA lesions and mutations that, in turn, can initiate carcinogenesis [39,49]. Biomarkers would be informative in early detection and prognosis of malignancy induced by UGS. Promising candidates, notably (1) estrogen-like and (2) 8-oxodG related metabolites highlighted here, appear worthy of validation in larger population based studies. Also, we caution that the malignant lesions included here included both UCC and SCC. Mixed urothelial cancer with squamous features, such as some cases in Group II, may have distinct biology compared to UGS related 'pure' SCC, and moreover the literature clearly indicates that UGS is a risk factor for SCC but not UCC [22–29]. Accordingly, investigation of larger populations may also facilitate the characterization of metabolites that characterize the discrete pathogenesis of SCC and UCC. These and related questions as noted above might also be addressed using informative cell lines, urine from UGS/SCC cases, and/or purified metabolites. Moreover, the novelty of metabolites excreted in urine during UGS suggests basic mechanisms for carcinogenesis in UGS-induced SCC, and maybe in other infection-related malignancies.

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Conflict of interest

The authors declare no conflicts of interest in preparing this article.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.01.018.

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Chapter 2

Infection with Opisthorchis felineus induces intraepithelial neoplasia of the biliary tract in a rodent model

CHAPTER 2. Infection with *Opisthorchis felineus* induces intraepithelial neoplasia of the biliary tract in a rodent model.

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The liver fluke *Opisthorchis felineus* is a member of the triade of epidemiologically relevant species of the trematode family Opisthorchiidae. Unlike *Opisthorchis viverrini,* group 1 agents and a major risk factor for cholangiocarcinoma, the carcinogenic potential of the infection with *O. felineus* is less clear. In this research we investigate if 1) *Opisthorchis felineus* also presented oxysterol-like metabolites similar to those detected in *O. viverrini* and 2) carcinogenic potential of the infection with *O. felineus*. To that we conducted both biochemical and histopathological investigations in hamster experimentally infected with *O. felineus*, a widely studied model of human opisthorchiasis and liver fluke infection-induced hepatobiliary disease as cholangiocarcinoma (CCA). The findings presented in this chapter support the inclusion of *O. felineus* in the Group 1 list of biological carcinogens. Two discrete lines of evidences emphasize the notion that *O. felineus* infection is carcinogenic. On one hand, novel oxysterol-like metabolites detected by LC-MS/MS in the egg and adult developmental stages of parasite, and in biological samples (bile, urine and sera) of liver fluke-infected hamsters exhibited marked similarity to oxysterol-like molecules known to *O. viverrini*. On other hand, histopathological analysis of liver sections from hamster infected with *O. felineus* revealed inflammation, severe periductal fibrosis and changes in the epithelium of biliary tract characterized as biliary intraepithelial neoplasia (BilIN), an established precancerous lesion that precedes intrahepatic CCA. The consonance of these findings revealed that *O. felineus* infection in rodent model induced precancerous lesions conducive to malignancy.

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ORIGINAL ARTICLE

Infection with *Opisthorchis felineus* induces intraepithelial neoplasia of the biliary tract in a rodent model

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Abstract

The liver fluke *Opisthorchis felineus* is a member of the triad of epidemiologically relevant species of the trematode family Opisthorchiidae, and the causative agent of opisthorchiasis felinea over an extensive range that spans regions of Eurasia. The International Agency for Research on Cancer classifies the infection with the liver flukes *Opisthorchis viverrini* and *Clonorchis sinensis* as group 1 agents and a major risk factor for cholangiocarcinoma. However, the carcinogenic potential of the infection with *O. felineus* is less clear. Here, we present findings that support the inclusion of *O. felineus* in the Group 1 list of biological carcinogens. Two discrete lines of evidence support the notion that infection with this liver fluke is carcinogenic. First, novel oxysterol-like metabolites detected by liquid chromatography-mass spectroscopy in the egg and adult developmental stages of *O. felineus*, and in bile, sera, and urine of liver fluke-infected hamsters exhibited marked similarity to oxysterol-like molecules known from *O. viverrini*. Numerous oxysterols and related DNA-adducts detected in the liver fluke eggs and in bile from infected hamsters suggested that infection-associated oxysterols induced chromosomal lesions in host cells. Second, histological analysis of liver sections from hamsters infected with *O.*

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felineus confirmed portal area enlargement, inflammation with severe periductal fibrosis and changes in the epithelium of the biliary tract characterized as biliary intraepithelial neoplasia, BilIN. The consonance of these biochemical and histopathological changes revealed that *O. felineus* infection in this rodent model induced precancerous lesions conducive to malignancy.

Abbreviations

BilIN	biliary intraepithelial neoplasia
CCA	cholangiocarcinoma
LC-MS	liquid chromatography-mass spectroscopy

Introduction

The liver fluke *Opisthorchis felineus* is one of the causative agents of opisthorchiasis. Formerly, *O. felineus* occurred primarily within the territory of the Russian Federation, especially in Western Siberia, the Ukraine, Byelorussia, Kazakhstan, and the Baltic countries (1). However, it is now increasingly seen in other European regions, including Italy where outbreaks of acute human infection have been reported recently (2–5). Worldwide, infection with *O. felineus* is responsible for about one in 10 cases of opisthorchiasis –1.6 million out of 17 million cases (6). This food-borne liver fluke is a member of to the trematode family Opisthorchiidae, which also includes the epidemiologically and clinically relevant species, *Opisthorchis viverrini* and *Clonorchis sinensis*. The International Agency for Research on Cancer (IARC) classifies the infection with *O. viverrini* and *C. sinensis* as group 1 carcinogens (7), definitive risk factors for cholangiocarcinoma (CCA) (8,9). The clinical manifestations and pathology induced by chronic infection with all of these opisthorchiid flukes are similar (10,11). However, the carcinogenic potential, physiology, molecular biology and mechanisms of host–parasite interaction are less well studied for *O. felineus* than *O. viverrini* and *C. sinensis*. There remains insufficient awareness of liver fluke infection with *O. felineus* as a problem for public health; nonetheless, infection with this species impact millions of people with severe morbidity and the geographical range of opisthorchiasis felinea continues to expand and to emerge in new locations (12).

Opisthorchis felineus has a complex life cycle involving three hosts; a gastropod snail and a cyprinoid fish serve as first and second intermediate hosts, respectively, and a mammalian piscivorous definitive host. Infection of the definitive host follows the consumption of fish contaminated with metacercariae. Bears, cats, dogs, foxes and people are all permissive definitive hosts where the parasite develops into adults within the intra- and extra-hepatic bile ducts and the gallbladder. Human infection is especially routine where consumption of smoked or uncooked fish is a dietary preference (5,13–15). The metacercaria excysts in the duodenum and the juvenile parasite ascends through the ampulla of Vater into the bile ducts, where the adult worm develops in 4–6 weeks. This liver fluke is a hermaphrodite, is long-lived and dwells within the biliary tract, feeding on epithelial cells, host blood and bile contents (11).

Opisthorchiasis felinea induces cholecystitis, cholangitis, gallbladder dysfunction, and hepatic abscess. Pathological changes that follow infection include chronic, proliferative cholangitis and pancreatic canaliculitis accompanied by tissue fibrosis (10,11). Available data indicate that the prevalence of liver cancer, largely diagnosed as CCA (16–18), is three times higher in liver fluke endemic regions of Western Siberia than in Russia at large (6). To date, there is a modest catalogue of supporting information for a role of infection with *O. felineus* as a risk for CCA (3,13,15), although this aspect has not been received sufficient investigation (6).

This epidemiological situation supports an association of infection-associated cancer related with chemical carcinogenesis along the lines of the pioneering report of Miller and Miller (19). To further investigate this phenomenon, here we undertook both biochemical and histopathological investigations in hamsters experimentally infected with *O. felineus*, a widely studied model of human opisthorchiasis and liver fluke infection-induced hepatobiliary disease including CCA (6,8,10,11). We characterized novel oxysterol-like metabolites using liquid chromatography-mass spectroscopy (LC-MS) in the adult and egg stages of the liver fluke and in bile, sera and urine of infected hamsters. The presence of diverse forms of DNA-adducts suggested that infection-associated oxysterols might be responsible for chromosomal lesions in host cells. In parallel, histopathological analysis revealed inflammation, severe periductal fibrosis and changes in the epithelium of biliary ducts identified as biliary intraepithelial neoplasia (BilIN), an established precancerous lesion that precedes intrahepatic CCA (20,21).

Materials and methods

Ethical statement

Procedures undertaken complied with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm. Syrian hamsters (*Mesocricetus auratus*) were purchased from the stock of the Puschino Animal Facility (Russia) and bred at the Animal Facility of the ICG SB RAS (RFMEFI61914X0005) (Russia). The hamsters were maintained according to protocols approved by the Committee on the Ethics of Animal Experiments of the Institute of Cytology and Genetics (Permit Number: 25 of 12.12.2014).

Parasites, hamsters and experimental design

Metacercariae of *O. felineus* were collected from naturally infected *Leuciscus idus* from the Ob River, Novosibirsk city, Western Siberia, and isolated from fish muscle tissues digested with pepsin–HCl overnight at 37°C. Territories where collection of the fish was undertaken were neither conservation areas nor private property, nor otherwise protected; hence, fishing permits were not required. *Leuciscus idus* is not considered endangered or rare, and the fishing methods complied with the Federal Law N166-F3 of 20.12.2004 (ed. 18.07.2011) 'Fishing and conservation of water bio-resources'.

Male Syrian golden hamsters aged 6–8 weeks were orally infected with 50 *O. felineus* metacercariae. The rodents were housed at three or four per cage under conventional conditions and received a stock diet and water *ad libitum*. Control non-infected hamsters (n = 4) and *O. felineus*-infected hamsters (n = 4) were necropsied 12 weeks after infection.

Sample collection and pathological studies

Hamsters were euthanized using carbon dioxide after which blood was collected by cardiac puncture. Blood was centrifuged at 3000*g* for 10 min at 4°C to obtain the serum. Ascorbic acid was added to 1 mg/ml (22–24) and the sera aliquoted and stored at –80°C. Urine was aspirated from the urinary bladder by syringe; ascorbic acid was added to 1 mg/ml. Urine samples were aliquoted and stored at –80°C. Bile was collected from the gallbladder by syringe aspiration; ascorbic acid was added to 1 mg/ml, aliquoted, and stored at –80°C.

Liver flukes recovered from livers of hamsters were thoroughly washed with sterile saline (0.9% NaCl) and incubated at 37°C, 5% CO_2 for

24 h in RPMI 1640 culture medium (Life Technologies) supplemented with 1×antibiotic/antimycotic (Sigma–Aldrich) and 1% glucose. Eggs laid *in vitro* from these cultured adult worms were collected by centrifugation of the culture medium, thoroughly washed with PBS and stored at –80°C. Soluble extracts of the flukes were prepared by sonication (5 × 5s bursts, output cycle 4, Vibra Cell, Sonics) in PBS supplemented with protease inhibitors [500 μM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 0.3 μM aprotinin, 10 μM E-64, 10 μM bestatin and 10 μM leupeptin] (M221, Amresco), followed by 30-min centrifugation at 10000 rpm and 4°C. The supernatant was collected and protein concentration determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Ascorbic acid was added to 1 mg/ml to the extracts; aliquots were stored at –80°C.

The livers were dissected and immersed in 10% buffered formalin (Biovitrum, Russia). After fixation overnight at 4°C, specimens were dehydrated through a graded series of ethanol to 100% ethanol, cleared in xylene, and soaked in melted paraffin. Thereafter, specimens were embedded in paraffin using Microm (Microm, UK) and 4-μm-thick sections were prepared using a microtome. For histopathological analysis, tissue sections were stained with hematoxylin and eosin (H&E) (11). The slides were examined under a light microscope (Axioskop 2 Plus; Zeiss, Germany), with magnification ×40, ×100 and ×400, and micrographs captured using a Nikon DS-5M-L1 camera.

Histopathology and Immunohistochemistry

Four μm-thick paraffin-embedded sections were prepared as above from both infected and non-infected hamsters for histopathological and immunohistochemistry analysis. Slides were stained with H&E and Masson's trichrome. For immunohistochemistry, formalin-fixed, paraffin-embedded tissue slides were screened for accumulation of p53 and proliferation (Ki-67) by using the streptavidin/biotin peroxidase and mouse monoclonal antibodies. The p53 protein was determined with clone DO-7 (DO-7, 1:50; Dako, Glostrup, Denmark), Ki-67 with clone MIB-1 (MiB1, 1:100; Dako). Briefly, slides were deparaffinised with xylene, rehydrated through a graded ethanol series, microwaved for 15 min in boiling citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0), and exposed to 3% hydrogen peroxide in methanol for 20 min. After blocking the endogenous peroxidase, slides were incubated in protein block solution (DakoCytomation). Thereafter, the samples were incubated overnight at 4°C with each of the primary antibodies and treated with secondary antibodies conjugated to a peroxidase-labelled polymer using the HISTOFINE system (Nichirei, Tokyo, Japan). Color development was performed using 3,3′-diaminobenzidine tetrahydrochloride as the substrate, after which slides were lightly counterstained with hematoxylin. Negative control sections were probed with BSA at 5% in PBS, devoid of primary antibody. Histopathological and histochemical changes were monitored by high-power microscopical examination at ×40, ×100 and ×400. Ki-67 and p53 nuclear staining was semi-quantified by estimating the percentage of positive nuclei per total number of nuclei of epithelial cells in the microscope field; they were classified as negative (≤5%), and positive (≥5%). Findings presented here accord with the criteria of the International Observer Agreement Guidelines for BilIN (20,21).

Sample preparation and LC-MS/MS

Samples were prepared and processed for LC diode array detection electron spray ionization mass spectrometry as described (25,26). Since methanol displays acceptable chromatographic performance in terms of separation and sensitivity, with short gradient times (27), this solvent was added to 20% by volume of the samples of serum, urine and bile from control and infected hamsters, and to extracts of *O. felineus* adult worms and of liver fluke eggs. Subsequently, bile samples were centrifuged (MiniStar silverline VWR, at 4000 rpm for 10 min after which the supernatant was collected; 25 μl of each sample was injected into LC-MS/MS. Duplicate samples were analyzed.

Higher performance liquid chromatography coupled with mass spectrometer (MS) was employed to investigate molecular species from the hamsters and liver flukes. The MS analysis was performed within a LTQ Orbitrap XL mass spectrometer (Thermo Fischer Scientific, Bremen, Germany), fitted with a ultraviolet (UV) photo diode array (PDA) detector. Analysis involved a single nucleosil C_{18}-column (250 mm × 4 mm internal diameter; 5 μm particle diameter, end-capped), proceeding at a flow rate of 0.3 ml/min. Eluates were monitored for 75 min, run with a mobile phase gradient of 0–5 min, 100% A; 5–10 min, linear gradient from 100% to 80% A, 10–15 min 80% A; 15–50 min, linear gradient from 80% to 40% A; 50–65 min, 40% A; 65–75 min, linear gradient from 40% to 100% B. Washing the column for 15 min with acetonitrile between each sample was undertaken in order to minimize carry-over and to stabilize the column. Data were collected in negative electrospray ionization negative mode scanning a mass to charge ratio (m/z) range of 50–2000. The capillary voltage of the electrospray ionization was 28 kW, capillary temperature was 310°C, flow rates of the sheath gas and auxiliary N_2 were set to 40 and 10 (arbitrary unit as provided by the software settings), respectively, and gas temperature was 275°C. At the outset, the workflow was undertaken using two samples for each biofluid, for example, sera from two groups: sera from control hamster followed by sera from infected hamsters, etc. The analyses of developmental stages of *O. felineus* were performed separately from biofluids in order to avoid possible contamination.

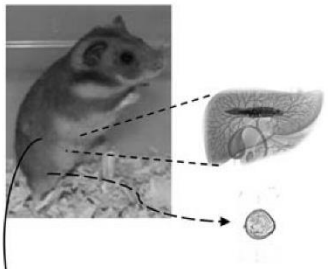
Results

Oxysterol-like molecules in developmental stages of *O. felineus*

Using LC-MS/MS, numerous oxysterol-like metabolites were detected in extracts of the adult and the egg stages *O. felineus* (Supplementary Figures S1 and S2 are available at *Carcinogenesis* Online). These extracts contained not only numerous novel and specific oxysterol-like metabolites, for example m/z 321 (m/z = mass to charge ratio) but also DNA-adducts (m/z 698) not detected in controls (Figure 1). The oxysterol-like metabolites of *O. felineus* were similar to those described for *O. viverrini*, for example m/z 385 and 438 (25), The proposed structures displayed ramification at C-17, that is discrete and variable chains linked to carbon 17 of the steroid ring, in similar fashion to bile acids and their conjugated salts (Supplementary Figure S2 is available at *Carcinogenesis* Online). Supplementary Figures S1 and S2, available at *Carcinogenesis* Online present predicted structures for the m/z species detected in *O. felineus* worms and eggs. These included bile acids, which constitute a large family of steroids carrying a carboxyl group on a side chain (m/z 425) (28), bile alcohols (m/z 362), which have similar products in bile acid biosynthesis or as final products, or free bile acids reconjugated in some species, as aldehydes (m/z 340, 356), glycine (m/z 425, 439) and sulfates (m/z 501, 557). This catalogue of oxysterols included molecular species compatible with the presence of derivatives of catechol estrogens and other components hydroxylated at the steroid ring, including at both C-2 and C-3 and respective oxidized 2,3-quinone (e.g. m/z 326, 461, 471). We speculate that effects of the individual oxysterol-like species on the pathophysiology during infection might be structure-dependent, with metabolic conversions resulting in a mixture of biologically active or inactive forms (25).

Parasite oxysterol-like molecules in biological fluids during infection

The chromatographic profiles of sera, urine and bile identified in controls and *O. felineus*-infected hamsters were compared and contrasted with extracts of the parasite (Figure 1 and Supplemenatry Figure S3, available at *Carcinogenesis* Online). The presence of oxysterol-related metabolites of parasite origin was unequivocally apparent. Figure 1 and Supplementary Figure S4, available at *Carcinogenesis* Online summarize the data and highlight similarities in metabolite species present both in *O. felineus* and the infected hamsters. Supplementary Figure S3, available at *Carcinogenesis* Online presents representative photo diode array and mass spectra chromatograms; samples were analyzed in duplicate and results presented as the average of the m/z



Developmental stages of *Opisthorchis felineus*

Adult form	
Metabolite (m/z)	200, 227, 237, 259, 265, 302, 307, 311, 325, 333, 339, 356, 362, 367, 385, 423, 424, 438, 461, 471, 500, 557, 599, 606
Eggs	
Metabolite (m/z)	117, 198, 274, 321, 397, 443, 564, 606, 677, 697, 716, 717, 723, 790, 816, 836, 933, 941, 949

Biofluids of *Opisthorchis felineus*-infected hamster

Bile		
Metabolite (m/z)	(from *O. felineus*) 339	*(from O. felineus and after metabolization)* 117, 226, 257, 430, 483, 617, 872, 908
Sera		
Metabolite (m/z)	(from *O. felineus*) 259, 325	*(from O. felineus and after metabolization)* 317, 539, 588, 667
Urine		
Metabolite (m/z)	(from *O. felineus* and after metabolization) 125, 166, 188, 231, 292, 322, 387, 467, 523	

Figure 1. Summary of findings from LC-MS/MS analysis of *Opisthorchis felineus* worms, eggs and biological fluids from hamsters infected with this liver fluke. The m/z species (mass to charge ratios) detected in adult liver flukes worms and the eggs of *O. felineus* are presented. M/z species common to bile and *O. felineus* are highlighted in green, and those common to sera and *O. felineus* in red. M/z species common to both *O. felineus* and hamster urine were not detected. Exclusive metabolites (m/z) from each one of the biofluids analyzed seemed to be associated with liver fluke infection, they may be directly related to compounds present in *O. felineus* that undergo metabolization after release from the parasite. Control samples were free of all m/z represented here.

obtained (Supplementary Table S1 is available at *Carcinogenesis* Online). As noted, a number of the metabolites were seen in both hamster biofluids and the liver flukes (Figure 1). The presence of specific DNA-adducts, for example m/z 617 in bile and m/z 588 in sera (Supplementary Figures S5 and S6, respectively are available at *Carcinogenesis* Online), suggested interactions of oxysterol-like metabolites and host cell chromosomal DNA. In addition, other parasite-derived metabolites, for example m/z 430 in bile and m/z 539 in serum (Supplemenatry Figures S5 and S6 are available at *Carcinogenesis* Online) might interact farther with host DNA. However, similar shared metabolites were not detected in urine of infected hamsters (Figure 1 and Supplementary Figure S7, available at *Carcinogenesis* Online).

BilIN associated with infection with *O. felineus*—BilIN 1/2/3

Sections of the livers of hamsters infected for 12 weeks revealed that the bile ducts were lined by cells with enlarged nuclei, exhibiting pseudo-stratification, hyperchromatism, loss of polarity, nuclear crowding, and mitotic figures with low-grade and intermediated-grade dysplasia (Figures 2–5). First, control, the epithelium of non-infected hamsters displayed a cuboidal to low-columnar phenotype with amphiphilic cytoplasm (Figure 2). By contrast, during infection, bile ducts were lined by epithelium with enlarged nuclei, pseudo-stratification, hyperchromatism, atypical hyperplasia and papillary duct lesion with atypia, loss of polarity, nuclear crowding and mitotic figures, low-grade dysplasia and some foci of moderate dysplasia, as described for BilIN (20,21). A transition from normal duct epithelium to an atypical lesion should suggest cancerization of the duct or ductile (Figure 2, panels B, B′ and C). The major histological changes in the gallbladder and extrahepatic bile ducts comprised adenomatous hyperplasia, epithelial hyperplasia and chronic inflammation. Masson's trichrome stains type I collagen a greenish/bluish hue, and is widely used to assess the degree of fibrosis. Severe periductal fibrosis was evident (Figure 3). Granulomatous inflammation, mononuclear cell and eosinophil infiltration in the portal area in response to entrapped parasite eggs in the periductal tissue and liver parenchyma also were evident (Figure 4A and B). These morphological alterations and phenotypic profile foci of BilIN were microscopically located in the intrahepatic large bile ducts. Thus, we observed BilIN-1 and 2, but also possible BilIN-3. However, BilIN-3, which occasionally shows p53 expression, was not readily evident. Additionally, bile duct epithelia showed high proliferative index (Ki-67) translating intraepithelial neoplasia (Figure 5A). Expression of p53 was not evident in the bile duct epithelium of infected hamsters (Figure 5B).

Discussion

Host–parasite interactions contribute to the molecular mechanisms of opisthorchiasis-associated pathology and clinical complications, including cholelithiasis and cholangiocarcinogenesis (8). Specific liver fluke-derived metabolites might directly damage the nuclear DNA of cholangiocytes, leading to malignant transformation. The introduction of enhanced LC-MS/MS approaches that require only minimal sample sizes and facile protocols has improved the analysis of conjugated bile acids (29),. Previously, we developed an LC-MS/MS method to identify steroid-based molecules in extracts of *O. viverrini* and *Schistosoma haematobium* and biofluids from human cases of urogenital schistosomiasis, involved in the estrogen metabolism (25,26,30). Using similar approaches here, we identified oxysterol-like metabolites from the egg and adult developmental stages of *O. felineus* with m/z, mass to charge ratio identical to those of *O. viverrini*. Moreover,

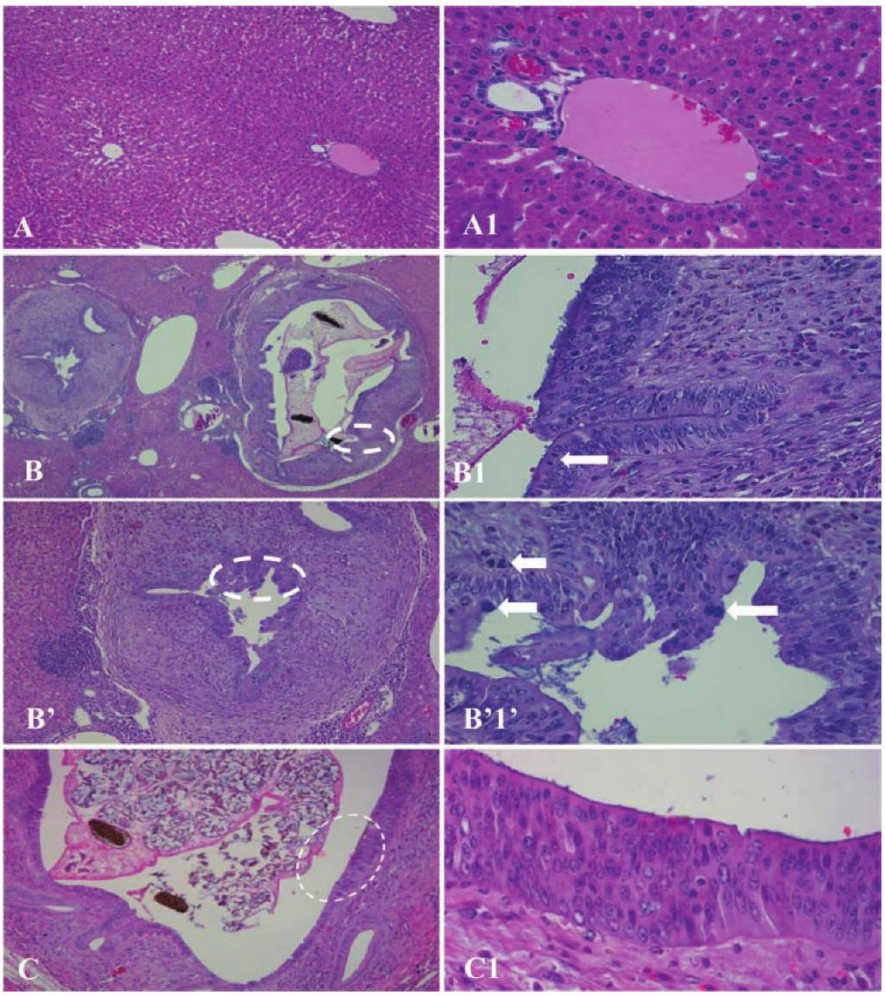


Figure 2. Biliary histological features observed in the liver biopsies from control, non-infected hamsters. (**A**) Normal portal unit with bile duct, hepatic arteriole, portal venule, and a clearly defined limiting plate (magnification ×200). The smaller or interlobular bile ducts are lined by cuboidal or low columnar epithelium. No evidence of inflammation (H&E staining); (**A1**) defines magnified area (magnification ×400) of normal portal unit. Biliary histological features observed in the liver biopsies of hamsters infected with *Opisthorchis felineus*. (**B, B′** and **C**) Biliary obstruction caused by the *O. felineus* worm with portal area enlargement (H&E staining) (magnification ×100). (**B1**) Dashed line defines magnified area (magnification ×100). (**A′**) dashed lines define magnified areas as (**B′1**) (magnification ×400) and (**C**) demonstrated the biliary obstruction caused by *O. felineus* with portal area enlargement. Bile ducts were lined by enlarged nuclei, with pseudo-stratification, hyperchromatism and some loss of polarity, nuclear crowding, mitotic figures (**C1, C′1**—arrows) and low-to moderate-grade of dysplasia. Evidence of flat or micropapillary dysplastic epithelium in the bile duct; these lesions are referred as biliary intraepithelial neoplasia (BilIN). (**C**) Epithelium lining a large intrahepatic bile duct displays flat hyperplasia with dysplastic changes (BilIN1/2). (**C1**) (magnification ×400), Note increased cellularity, modestly increased pseudo-stratification, and nuclear irregularities including variation in size and polarity, cytologic atypia including presence of nucleoli and loss of polarity (BilIN2).

the analysis of sera, bile and urine of hamsters infected with *O. felineus*, and comparisons with chromatograms from adult and egg developmental stages, revealed metabolites with the same or similar m/z, for example metabolites with m/z 325 for sera and 339 for bile.

Similar to *O. viverrini*, analysis of *O. felineus* revealed the presence of oxysterols of diverse forms: bile alcohols, bile aldehydes and reconjugated as glycine or sulfates. Catechol forms (e.g. m/z 461) also were identified (Supplementary Figure S1 is available at *Carcinogenesis* Online). Oxysterols observed in the liver fluke *O. felineus* might, at least in part, arise from nonenzymatic reaction with oxidative free radical-like oxygen and nitrogen species. It is also feasible that they could originate enzymatically as products of cytochrome P450 family enzymes. Indeed, a sole member of the cytochrome P450 family of genes is known from the genome of *O. felineus* (31,32). Chronic inflammatory responses to *O. viverrini* generate reactive oxygen species and reactive nitrogen species (33), which might react with biomolecules within the inflammatory tissue such as the bile ducts during opisthorchiasis felinea.

Biliary tract epithelia and surrounding tissue of hamsters infected with *O. felineus* displayed forms of BilIN. BilIN has been defined as a precursor lesion of invasive adenocarcinoma in the biliary tract (20,21), and represents the multistep process in carcinogenesis. BilIN represents a spectrum of proliferative and/or cytological atypical lesions of the large intrahepatic bile ducts, considered a major transformation within the pathway leading to intrahepatic CCA. BilIN lesions are characterized by a flat or micropapillary dysplastic epithelium in the bile ducts and, according to international guidelines, have been used

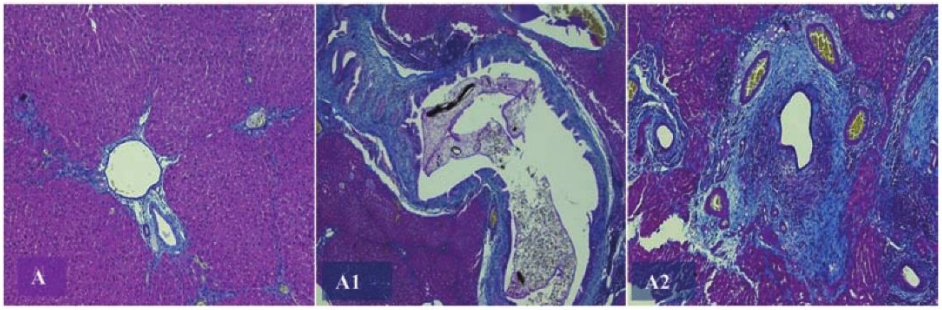


Figure 3. (**A**) Normal portal tract free of fibrosis (stained with Masson's trichrome) (magnification ×40). (**A1** and **A2**) Masson's trichrome stained tissues showing massive peri-portal fibrosis (region stained blue, magnification ×100). Fibrosis is not only the result of necrosis, collapse, and scar formation but also results from derangements in the synthesis and degradation of matrix by injured mesenchymal cells that synthesize the various components of the matrix.

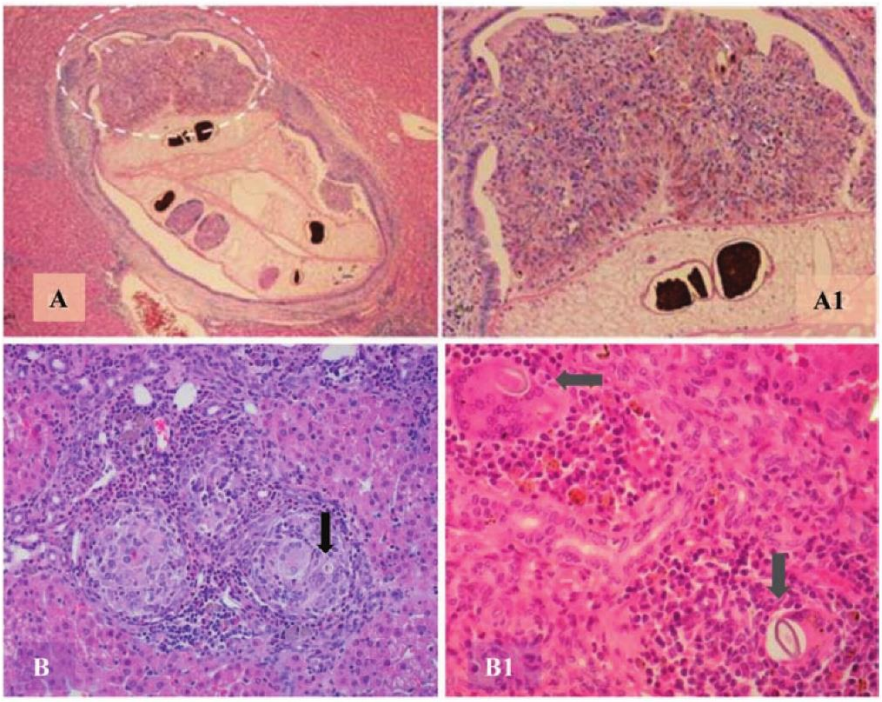


Figure 4. (**A**) Epithelioid granuloma with multinucleated giant cells, lymphocytes, and eosinophils in portal area, occasionally surroundings eggs. Biliary duct obstruction caused the presence of an adult *O. felineus* liver fluke (magnification ×40). (**A1**) Dashed line defines magnified area. Evidence of egg granulomata identified by white arrows surrounding by inflammatory cells (magnification ×100). (**B**) Granulomas with multinucleated giant cell (magnification ×100). (**B1**) Mononuclear and eosinophilic cell infiltration in portal regions and multinucleated giant cells surroundings eggs (arrow) (magnification ×400).

instead of traditional biliary dysplasia. These pre-malignant lesions are classified into three grades: BilIN-1 (low-grade lesion), BilIN-2 (intermediate-grade lesion) and BilIN-3 (high-grade lesion). Here, BilIN-1 and BilIN-2 lesions were characterized unequivocally in the *O. felineus*-infected hamsters. The findings strongly suggested that infection with *O. felineus* is associated with intracellular mechanisms that eventually trigger neoplastic transformation of cholangiocytes, and promote biliary carcinogenesis. Multifocal BilIN were clear histopathological features observed in this rodent model. These findings support earlier observations that *O. felineus* might induce CCA, at least in hamsters (11). It is reasonable to speculate that the pathobiology of *O. felineus* and *O. viverrini* infections are similar (15). Both species of *Opisthorchis* injure the host through direct physical and/or immunopathological processes and indeed damage from infection with *O. felineus* is even more marked than *O. viverrini* (10).

Atypical hyperplasia and dysplasia of the epithelium of the bile duct and egg granulomas in the periductal tissues and liver parenchyma are potential precancerous lesions. Eggs may extravagate into the adjacent parenchyma and incite a granulomatous response and infiltration of lymphocytes, plasma cells and eosinophils, and fibrosis. We hypothesize that parasite-specific oxysterols contribute to the granuloma formation and progression, given the broad range of these metabolites identified in the eggs. The hypothesis that the eggs provoke granulomatous inflammation also is supported the findings in bladder cancer lesions induced by urogenital schistosomiasis where schistosome eggs at the nidus of the granulomatous lesions generate estrogen-like molecules that damage the DNA of urothelial cells.

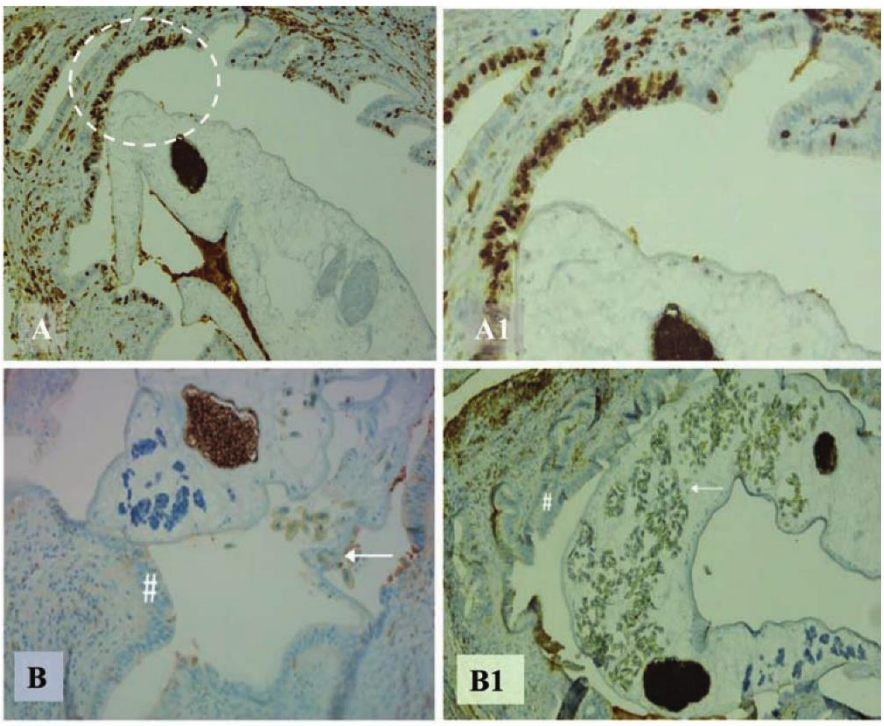


Figure 5. Immunohistochemistry to reveal expression of Ki-67 and p53 in liver from hamsters infected with the liver fluke *Opisthorchis felineus* (**A**) (magnification ×100), Epithelium of bile ducts with high proliferative index (Ki-67) translating intraepithelial neoplasia (**A1**, magnification ×400x). (**B** and **B1**, magnification ×100), expression of p53 was not observed in non-neoplastic epithelium of the bile ducts, as well as in BilIN-1 and BilIN-2 (#). Eggs of *O. felineus* (arrow).

Inflammatory responses against schistosome eggs in the wall of the urinary bladder represent early events in the pathogenesis of urogenital schistosomiasis (34).

We posit that carcinogenesis associated with chronic infection with *O. felineus* mimics malignant transformation induced by infection with *O. viverrini* and *C. sinensis*. The findings presented here support this hypothesis. The findings revealed, first, the presence of cholesterol-derived metabolites of liver fluke origin. Second, they revealed that these oxysterol-like metabolites exhibited striking similarity to those of *O. viverrini*. Third, they revealed unequivocal signals of BilIN-1 and BilIN-2. Oxysterols are products of oxidation of cholesterol that arise through enzymatic (P450) or non-enzymatic processes. Oxysterols display mutagenic, genotoxic, pro-oxidative and pro-inflammatory properties that can contribute to malignancy. Associations between oxysterols and the development and progression of cancer of colon, lung, breast and bile ducts have been proposed (35,36). As with infection with *O. viverrini* and also during urogenital schistosomiasis (8,37), we speculate that *O. felineus* produces oxysterols excreted to the biliary system where they may cause lesions in chromosomal DNA of the cholangiocytes lining the biliary tree, which result in due course in BilIN.

It has been generally accepted that CCA tumors in this hamster model occur only as a result of a combined action of infection with the liver flukes, *O. felineus*, *O. viverrini* and *C. sinensis* and exposure to *N*-nitrosodimethylamine (11,38,39). However, the nature and occurrence of precancerous lesions during liver fluke infection has remained poorly understood. Here, we assessed the expression of Ki67 and p53, and presence of intraepithelial bile duct neoplasia and severe fibrosis. Together these data indicate that precancerous changes occur in hamsters without exposure to an exogenous carcinogen such as *N*-nitrosodimethylamine. Concerning how CCA develops, the role of liver flukes has been assigned as a tumor cell growth promoter, whereas the role of *N*-nitrosodimethylamine is a mutagenic cancer inducer. By contrast, we now demonstrate the presence of parasite-specific oxysterol metabolites conjugated with DNA bases presumably derived from host tissues. The presence of these adducts provides cogent indirect evidence of both mutagenic *and* carcinogenic potential of infection with *O. felineus*. Thus, the role for liver flukes is not restricted only to promotion of chronic inflammation, establishment of conditions favorable to the promotion and proliferation of incipient cancer cells, and tumor growth, but now can be seen to also include mutagenesis capable of initiation of the malignant transformation. This represents the key and notable advance provided by these new findings.

To date, the International Agency for Research on Cancer categorizes infection with *O. felineus* as a group 3 carcinogen, i.e. there is insufficient evidence yet for its classification as carcinogenic, unlike the situation with the closely related liver flukes *O. viverrini* and *C. sinensis*, and the blood fluke *S. haematobium* which are group 1 carcinogens—definitely carcinogenic in humans. However, our findings highlight the need for reconsideration of this classification with respect to infection with *O. felineus* (7). To summarize and conclude, we report that *O. felineus* flukes secreted/excreted oxysterol-like molecules into the bile, which in turn may react with host chromosomal DNA to form apurinic sites causing error-prone base excision repair leading to mutations and, ultimately, initiate carcinogenesis (25,28,40). We also document clearly that infection with *O. felineus* induces BilIN. The consonance of findings that demonstrate both the presence of these metabolites and of BilIN-1 and BilIN-2 indicates that *O. felineus* infection induces neoplastic transformation of cholangiocytes. Hence, this infection can be expected to promote growth

of biliary cancers. Deeper investigation is warranted in order to decipher relationships between liver fluke oxysterols and malignancy, including during opisthorchiasis felinea in humans.

Supplementary Material

Supplementary data are available at *Carcinogenesis* online.

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Chapter 3

Infection with carcinogenic helminth parasites and its production of metabolites induces the formation of DNA-adducts

CHAPTER 3. Infection with carcinogenic helminth parasites and its production of metabolites induces the formation of DNA-adducts

Authors: Maria João Gouveia, Paul J. Brindley, Gabriel Rinaldi, Fátima Gärtner, José Manuel Correia da Costa, Nuno Vale.

Infections classified as group 1 biological carcinogens include the helminthiases caused by *Schistosoma haematobium* and *Opisthorchis viverrini*. As presented in previous chapters, we have postulated that these parasites release metabolites including oxysterol and estrogen-like metabolites that might interact with host DNA. During the work presented in this Chapter we evaluate the role of CYP450 in the formation of metabolites associated with helminth infections and its interactions with DNA. For that purpose, we performed *in vitro* assays using similar compounds to parasitic metabolites reported, and exogenous DNA in the presence or absence of CYP1A1, 2E1 and 3A4. The reaction products identified by LC-MS/MS shown the formation of similar metabolites previously associated with schistosomiasis and opisthorchiasis and DNA-adducts proving its ability to interact with DNA. Apparently, CYP450 was not essential for the formation of these metabolites. The formation of DNA adducts *in vitro* supports the hypothesis that chemical carcinogenesis might be involved in promoting helminthiases-associated carcinogenesis.

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Infection with carcinogenic helminth parasites and its production of metabolites induces the formation of DNA-adducts

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Abstract

Background: Infections classified as group 1 biological carcinogens include the helminthiases caused by *Schistosoma haematobium* and *Opisthorchis viverrini*. The molecular mediators underlying the infection with these parasites and cancer remain unclear. Although carcinogenesis is a multistep process, we have postulated that these parasites release metabolites including oxysterols and estrogen-like metabolites that interact with host cell DNA. How and why the parasite produce/excrete these metabolites remain unclear. A gene encoding a CYP enzyme was identified in schistosomes and opisthorchiids. Therefore, it is reasonable hypothesized that CYP 450 might play a role in generation of pro-inflammatory and potentially carcinogenic compounds produced by helminth parasites such as oxysterols and catechol estrogens. Here, we performed enzymatic assays using several isoforms of CYP 450 as CYP1A1, 2E1 and 3A4 which are involved in the metabolism of chemical carcinogens that have been associated with several cancer. The main aim was the analysis of the role of these enzymes in production of helminth-associated metabolites and DNA-adducts.

Method: The effect of cytochrome P450 enzymes CYP 1A1, 2E1 and 3A4 during the interaction between DNA, glycocholic acid and taurochenodeoxycholate sodium on the formation of DNA-adducts and metabolites associated with urogenital schistosomiasis (UGS) and opisthorchiasis was investigated *in vitro*. Liquid chromatography/mass spectrometry was used to detect and identify metabolites.

Main findings: Through the enzymatic assays we provide a deeper understanding of how metabolites derived from helminths are formed and the influence of CYP 450. The assays using compounds similar to those previously observed in helminths as glycocholic acid and taurochenodeoxycholate sodium, allowed the detection of metabolites in their oxidized form and their with DNA. Remarkably, these metabolites were previously associated with schistosomiaisis and opisthorchiasis. Thus, in the future, it may be possible to synthesize this type of metabolites through this methodology and use them in cell lines to clarify the carcinogenesis process associated with these diseases.

Principal conclusions: Metabolites similar to those detected in helminths are able to interact with DNA in vitro leading to the formation of DNA adducts. These evidences supported the previous postulate that imply helminth-like metabolites as initiators of helminthiases-associated carcinogenesis. Nonetheless, studies including these kinds of metabolites and cell lines in order to evaluate its potential carcinogenic are required.

Keywords: (max. 10): Helminth infection, Malignancy, Schistosomiasis, Opisthorchiasis, Cytochrome P450, Oxysterol

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Background

More than 20% of cancers in the developing world are caused by infections, including helminthiases [1]. In addition to the direct impact on the development, health, and prosperity of populations in endemic regions, chronic infection with the liver fluke *Opisthorchis viverrini* and the blood fluke *Schistosoma haematobium* leads to cholangiocarcinoma (CCA), bile duct cancer, and squamous cell carcinoma (SCC) of the urinary bladder, respectively. The chronic infection with these helminths is recognized by International Agency for Research on Cancer (IARC) as a definitive cause of cancer [2].

These helminths produce and excrete metabolites, including estrogens and oxysterols, that appear capable of oxidation of host DNA, in turn leading to the formation of depurinating DNA adducts and mutations in the genome of adjacent tissues acting as initiators of carcinogenesis [3–7]. The high-performance liquid chromatography coupled with mass spectrometry (LC-MS/MS) analysis of urine from individuals with urogenital schistosomiasis (UGS) and bladder cancer revealed the presence of specific metabolites which may represent biomarkers for diagnosis and prognosis of SCC [6]. These metabolites were also identified in developmental stages of *S. haematobium* and *Opisthorchis viverrini* [5–8]. Chronic infection with the liver fluke, *Opisthorchis felineus* may mimic the malignant transformation induced by *O. viverrini* infection producing metabolites that interact with host DNA. In addition, several DNA adducts were detected in biofluids from hamsters experimentally-infected with *O. felineus*. These findings may support the inclusion of the infection with the liver fluke *O. felineus* as group 1 carcinogens by the IARC [5].

The CYP450 enzymes are involved in several biological processes such as biosynthesis of estrogen, conversion of cholesterol into bile acids and its biotransformation [9, 10]. These enzymes include the CYP450 family members CYP 1A1, 2E1 and 3A4, involved in the metabolism of chemical carcinogens, associated with breast and endometrium cancer in humans [11]. A gene encoding a CYP enzyme was identified in schistosomes and opisthorchiids [12–14]. Accordingly, this family of enzymes might play a role in generation of pro-inflammatory and potentially carcinogenic compounds produced by helminth parasites such as oxysterols and catechol estrogens. The role of CYP 450 enzymes in the physiology and biochemistry of these flukes is less well understood. However, it might contribute to cell survival, drug resistance, maintenance and evolution of the host-parasite relationships [13]. In case of *O. felineus* the function of CYP450 has been linked to the excretory system of the parasite and possibly to metabolism and detoxification as biotransformation of endogenous substrates [13]. Nevertheless, it remains unclear whether trematode CYP enzymes catalyze the synthesis of proinflammatory and potentially carcinogenic compounds, e.g. oxysterols-like and catechol-like estrogen quinone-like metabolites known to be released by these flukes [6–8].

Previously, we have hypothesized that helminth-derived metabolites may be able to interact with host DNA [3, 4]. Here we conducted in vitro assays in order to evaluate the role of CYP450 in the formation of carcinogenic metabolites [5–8]. Due to difficulties in isolating the metabolites detected on developmental stages of the parasites or biofluids of infected individuals, we decided to use commercially-available compounds, i.e. glycocholic acid and taurochenodeoxycholate sodium. Thus, we exposed exogenous DNA to glycocholic acid and taurochenodeoxycholate sodium in the presence or absence of CYP 450. Subsequently, we identified the reaction products by liquid chromatography-mass spectrometry (LC-MS/MS).

Materials and methods

Chemicals

Acetonitrile (ACN) and formic acid (HF), HPLC grade, were obtained from Merck (Darmstadt, Germany). Glycocholic acid hydrated (G2878-100MG), calf thymus DNA (D1501-100MG), nicotinamide adenine dinucleotide phosphate (NADPH, N7506-25MG), dimethyl sulfoxide (DMSO, D-5879) and CYPExpress™ 1A1 (MTOXCE1A1-250MG), 2E1 (MTOXCE2E1-250MG) and 3A4 (MTOXCE3A4-250MG) were purchased from Sigma/Merck (Sintra, Portugal). Taurochenodeoxycholate sodium (20275) was purchased from Cayman Chemical (Ann Arbor, Michigan, USA).

CYP450 activity, interaction of precursor metabolites with DNA and formation of DNA adducts

The structures of compounds glycocholic acid (**1**) and taurochenodeoxycholate sodium (**2**) are shown in Fig. 1. Stock solutions of both compounds were prepared in DMSO. The compounds at final concentration of 100 μM were incubated with CYP1A1, CYP2E1 and CYP3A4 (0.2 μM) in the presence of 1.4 μM of NADPH and calf thymus DNA (3 mM) in 67 mM Na-K phosphate buffer (pH 7.2), in a total volume of 200 μL at 37 °C for 72 h [15, 16]. A control reaction was prepared as described above but without CYP450 enzymes. In addition, a well containing the matrix of reactions was also prepared with NADPH, calf thymus and Na-K buffer. Aliquots of the reaction mixture were collected at 24 and 72 h after the start of the reaction. The reaction was stopped by addition of two volumes of ethanol (EtOH) to precipitate DNA, which was recovered by centrifugation. Thereafter, the supernatant was subjected to analysis by LC-MS/MS. It should be noted that data originated from matrix sample were subtracted (in LC/MS) to eliminate its influence.

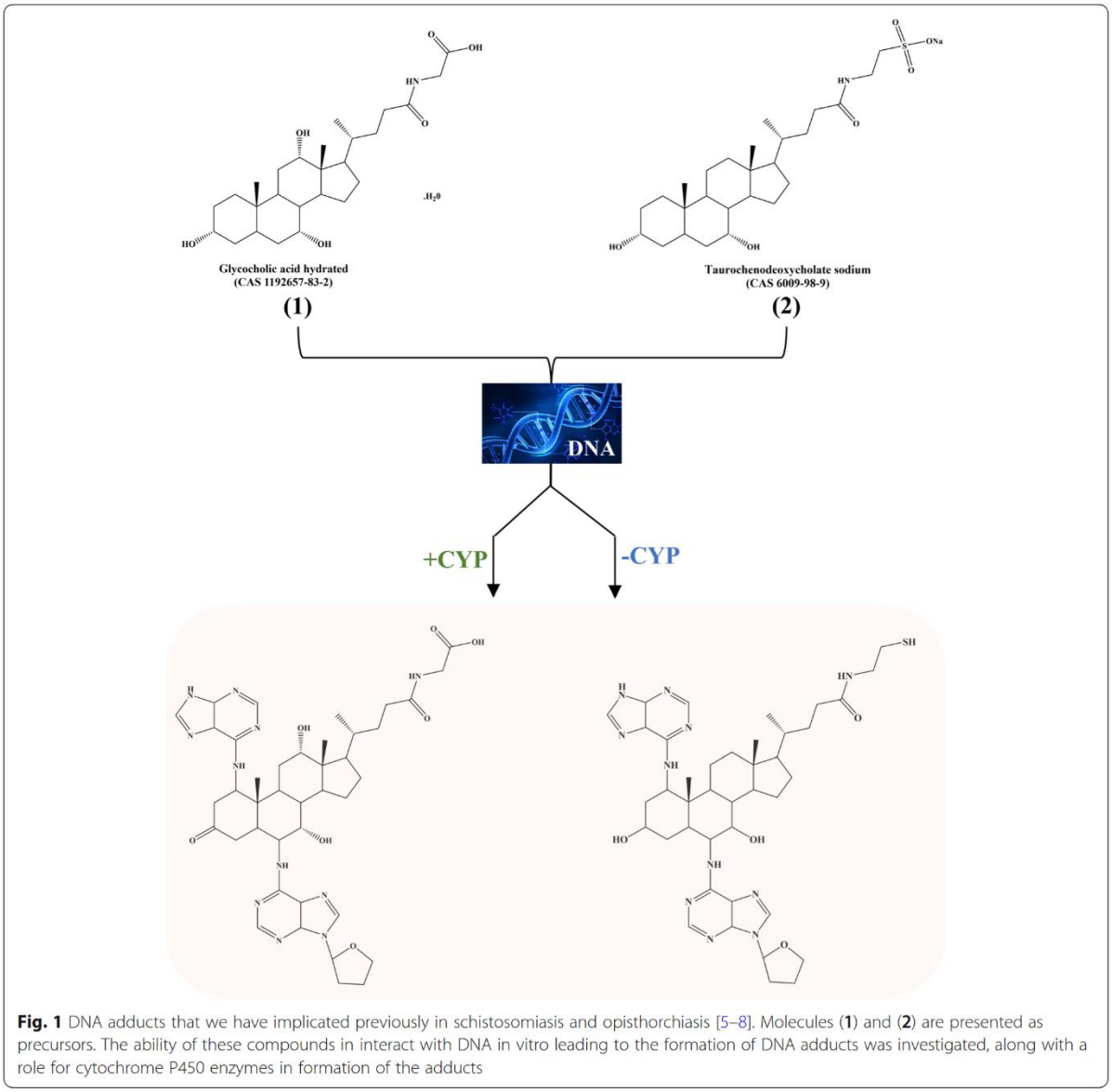

Fig. 1 DNA adducts that we have implicated previously in schistosomiasis and opisthorchiasis [5–8]. Molecules (**1**) and (**2**) are presented as precursors. The ability of these compounds in interact with DNA in vitro leading to the formation of DNA adducts was investigated, along with a role for cytochrome P450 enzymes in formation of the adducts

Detection of metabolites and DNA-adducts by liquid chromatography mass spectrometry (LC-MS/MS) analysis

Detection and identification of metabolites and related DNA-adducts by LC-MS/MS was undertaken using an LTQ Orbitrap XL mass spectrometer (Thermo Fischer Scientific, Bremen, Germany), fitted with an ultraviolet (UV) photo diode assay (PDA) detector. Analysis of aliquots involved a single injection of 20 μL with an ACE Equivalence 5 C_{18} (75 mm X 3 mm i.d.) column. The mobile phase consisted of 1% HF in water (A)/ACN (B) mixtures. Elution proceeded at a flow rate of 0.5 ml/min. Eluates were monitored for 10 min, run with a mobile phase gradient started with 80% A and 20% B. At that point, B was increased linearly to 55% over 5 min. The gradient was returned to the starting proportion at 9 min and equilibrated for one minute. Data were collected in positive electrospray ionization (ESI). The capillary voltage of the ESI was 28 kV, and its temperature was 310 °C, flow rates of the sheath gas and auxiliary gas (N_2) were set to 40 and 10 (arbitrary units as provided by software settings), respectively, and gas temperature was 275 °C.

Results

Formation of glycocholic acid and taurochenodeoxycholate sodium metabolites and DNA adducts is independent of CYP 450

The chromatograms and ratio mass/charge (m/z) obtained by LC-MS/MS analysis are shown in Additional files 1 and 2. With regard to the starting compounds, the precursor glycocholic acid (**1**) with molecular mass [M + H] 466.32 was identified in all aliquots analyzed (Additional file 3). Additionally, product spectra as ratio mass/charge (m/z) 338 $[M\text{-}3H_20\text{-}C_2H_4NO_2 + H]^+$ and 412 $[M\text{-}3H_20 + H]^+$ were identified as its metabolites, as previously described [17]. In contrast, taurochenodeoxycholate sodium (**2**) (m/z 522.28) was not detected.

Through the analysis of the data obtained for aliquots (Additional file 1), it was possible to observe that sample (+CYP) at 24 h and control (−CYP) at 72 h included more metabolites than (+CYP) at 72 h and (−CYP) at 24 h. Apparently, the number of metabolites on samples decreased through the course of reaction. The opposite effect was observed in the controls, i.e. at 24 h there were fewer compounds than at 72 h; 43 vs 77. For the samples (+CYP) during the course of the reaction, the number of compounds decreased, suggesting that the reaction in the presence of CYP450 was faster but might lead to some transformation and, in turn, result in fewer products. By contrast, in controls (−CYP) the number of compounds slightly increased during the reaction which suggested that reaction in the absence of CYP450 might be slower, and lead to the formation of an elevated number of compounds. In turn, this might indicate that the chemical transformation was less evident in controls (−CYP) (Additional file 1).

Nevertheless, some of the metabolites detected were common to 1) all aliquots; 2) samples (+CYP) (e.g. samples at 24 and 72 h); 3) control (−CYP) (e.g. control at 24 and 72 h); 4) samples or control at same time of reaction (e.g. sample and control at 24 h); 5) samples (+CYP) or control (−CYP) on different time of reaction (e.g. sample at 24 and control at 72 h); 6) to three different aliquots (e.g. sample at 24, controls at 24 and 72 h) (Additional file 2). Generally, the generated compounds were mostly metabolites of precursors in oxidized or catechol form and DNA-adducts (Additional files 2 and 3). Indeed, samples and controls at 24 h included several common metabolites that correspond not only to oxidized metabolites (e.g. m/z 628.30, Additional file 3) but also DNA-adducts (e.g. 718.41, Additional file 3). The highest number of DNA adducts was observed in the sample (+CYP) at 24 h; however, some of these DNA-adducts were also detected on control (−CYP) at 24 h. Similarly, some compounds either in oxidized form or as DNA-adducts detected in samples (+CYP) at 24 h were also present in controls (−CYP) at 72 h (e.g., m/z 734.40 and 801.43 Additional file 3). Since metabolites and related DNA-adducts were detected either in samples (+CYP) or controls (−CYP) at the same or at a different time of reaction, we hypothesize that their formation was independent of the presence of CYP450. Notably, the compounds that were common to three samples, (+CYP) at 24 and (−CYP) at 24 and 72 h were also DNA adducts (e.g., m/z 566.34). Curiously, in comparison (+CYP) at 24 and 72 h and (−CYP) at 72 h and (+CYP) 24 h and 72 h, most of the common compounds were fragments of precursors (e.g., m/z 403.27, Additional file 3).

Comparing all exclusive metabolites obtained for all aliquots, we observed that controls (−CYP) 72 h had the most elevated number (56) but also the lowest at 24 h (10) (Fig. 2). It was expected that the number of compounds would be higher in samples (+CYP) than in the controls (−CYP). However, as noted above, this could suggest that the formation of metabolites and DNA adducts was independent of CYP450, and that most of the metabolites form through a chemical metabolization that occurs in the reaction mixture, and not by enzymatic catalysis (Fig. 2).

Generally, the exclusive compounds were mainly metabolites of precursors of glycocholic acid and taurochenodeoxycholate sodium, and most of them were in oxidized or catechol form [(e.g. m/z 541.21 at S24 h (+CYP); m/z 509.24 at C72 h (−CYP)]. Interestingly, at C24 h (−CYP) we did not observe exclusive metabolites in oxidized or catechol forms (Additional file 4). All aliquots except C72 h (−CYP) presented DNA adducts [e.g. m/z 616.34 at S24 h (+CYP); m/z 899.43 at C24h (−CYP); m/z 598.75 at S72 h (−CYP)]. Regarding the common metabolites, aliquots S24 h (+CYP) and C24 h (−CYP) shared not only metabolites in oxidized form as well as DNA-adducts (e.g. m/z 628). Similarly, this was observed on S24 h (+CYP) and C72h (−CYP) (e.g. m/z 743.40), and S72 h (+CYP) and C72 h (−CYP) (e.g. m/z 612.44) (Fig. 2; Additional file 4). Additional files 3 and 4 present the common and exclusive postulated structures of the metabolites and cognate DNA-adducts.

The metabolites and DNA-adducts are similar to those identified during schistosomiasis and opisthorchiasis

In order to test if the in vitro assay leads to the synthesis of metabolites related to schistosomiasis [6] and opisthorchiasis [5, 7], we compared the current data with published findings [5–8]. The identities of common compounds were confirmed by comparison of their mass spectra and molecular mass (Fig. 2). Indeed, some metabolites and DNA-adducts synthesized during the present assays (e.g. m/z 600, 718, 804, Figs. 2 and 3) had been previously associated with these helminth infections [5, 7]. In addition, some metabolites detected here were identified as oxidized forms [M + 16] of those previously described for helminthic infection [5, 6, 7] (Figs. 2 and 3).

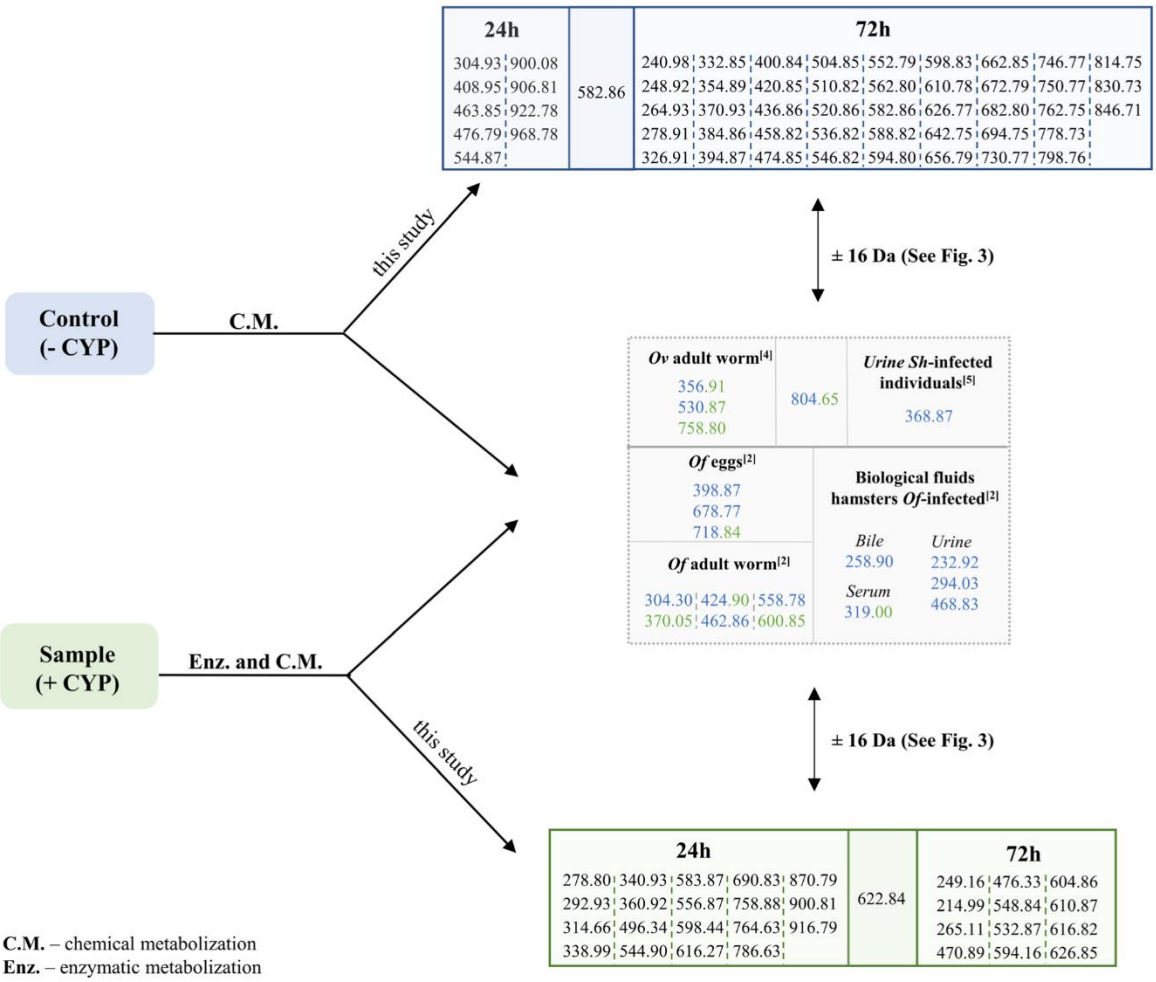

Fig. 2 Exclusive m/z detected in control (–CYP) and samples (+CYP) during the course of the reaction. Several compounds that had been detected previously within developmental stages of parasites and biofluids from mammalian hosts were synthesized in vitro in this study

Interestingly, most of these similar compounds were not DNA-adducts, but were precursor metabolites.

Additionally, most of these compounds were detected on control (–CYP) or were common to control (–CYP) and samples (+CYP) suggesting that CYP450 was not critical to produce electrophilic metabolites observed on flukes and infected individuals (Figs. 2, 3). Based on these findings, we could hypothesize that infection with the liver fluke might have a potential role in the formation of DNA-adducts.

In addition, during this in vitro study, it was possible to synthesize a new family of metabolites of glycocholic acid (**1**) and taurochenodeoxycholate sodium (**2**), as well as to demonstrate that these metabolites are able to interact with DNA forming DNA-adducts. It should be noted that some the metabolites and DNA adducts synthesized here correspond to those previously related to schistosomiasis and opisthorchiasis. Thus, we consider that data presented here reinforce the notion that this kind of compounds and the helminth-derived metabolites, are capable to interact with host DNA and lead to the formation of DNA-adduct. Therefore, these evidences support, at least in part our postulated [3, 4].

Discussion

In this study, we performed an in vitro assay to investigate the generation of some of the metabolites and related DNA-adducts previously identified in the context of opisthorchiasis, schistosomiasis and their infection-associated cancers [5, 6, 8]. The precursors, glycocholic acid (**1**) and taurochenodeoxycholate sodium (**2**), were chosen based on previous data that demonstrated cognate DNA-adducts in developmental stages of parasites and in biofluids of human cases and/or rodent models of these helminth infections [5–8]. Using in vitro

Fig. 3 Mass/ratio (m/z) of metabolites and DNA adducts synthesized in vitro, and previously related to opisthorchiasis and schistosomiasis which undergo hydroxylation (± 16 Da)

assays, we confirmed the ability of these compounds to interact with DNA leading to the formation of DNA adducts.

Bile acids exhibit a diverse spectrum of biological activity, being able to stimulate proliferation and tumor invasiveness, inhibit apoptosis and modify the promoter functions of genes involved in DNA synthesis, repair, and oxidative stress [18, 19]. Bile acids are associated to several carcinogen models, e.g. glycocholic acid was high in urine of people with hepatocellular carcinoma [20]. Additionally, in human esophageal cell lines, acid and bile salts, including glycocholic acid (**1**), caused elevated DNA damage [20]. CYP450 enzymes are involved in the generation of most chemical carcinogens that induce genotoxicity and DNA damage [10, 21, 22]. CYP450 play a critical role both in estrogen formation and its subsequent metabolism, namely, CYP1A1 and CYP3A which catalyze the hydroxylation preferentially at the 2-position, whereas CYP1B1 catalyzes the hydroxylation almost exclusive at the 4-position [23], leading formation of catechol estrogen quinone form that can react with DNA to form predominantly depurinating adducts.

We have previously hypothesized a similar pathway for the genesis of parasite-derived metabolites [4]. Here, we investigated the role of these enzymes in activation and/or oxidation of parent compounds. Although CYP450 enzymes might be involved in formation of some metabolites detected in samples (+CYP), apparently, they were not crucial during the metabolization of these compounds to form DNA-adducts, since they were detected in control aliquots (−CYP). The metabolites and oxidized forms detected here were probably generated by

non-enzymatic autoxidation process [24, 25]. Nonetheless, some of oxidized metabolite were observed in samples (+CYP). Therefore, we cannot rule out that these enzymes play a role in the formation of oxidized metabolites since involvement of CYP450 in the oxidation of several compounds have been reported in previous study [26]. Apparently, previous activation is not necessary for interaction with DNA. Notably, the metabolites and DNA adducts that were previously described [5–8], and synthesized here for use in our in vitro assay, were generated in absence of P450. This suggests that the formation of these metabolites also might be independent of the parasite CYP450.

Carcinogenesis is a complex process in which normal cell growth is modified due interaction of multiple factors [27]. Malignant transformation follows a sequence of steps that include a pathogenic *stimulus* (biological and/or chemical) followed by a chronic inflammation, in turn provoking fibrosis and changes in the cellular microenvironment arising in a pre-cancerous niche [28]. In the cases of schistosomiasis and opisthorchiasis, we have postulated that these pathogens provide biological and chemical *stimuli* through parasite-derived metabolites, e.g. oxysterols and catechol estrogens, that can interact with host DNA to trigger a cascade of events ultimately leading to cancer [3–8]. Cholesterol, oxysterols and estrogens are closely related steroids [29]. These trematode parasites likely produce these steroid-like compounds for endogenous physiological and reproductive processes, but they| may also have evolved in concert with the host-parasite relationship. Oxysterols and/or catechol estrogens of trematode origin and/or precursors modified as the consequence of opisthorchiasis or UGS [2–8, 30] appear capable of modulating host metabolic pathways of steroid hormones and bile acids [31], and are potential initiators of carcinogenesis given that these metabolites are genotoxic in other systems [32]. Since these infection-related initiators of cancer and/or promoters might need to be metabolized for activation, their carcinogenicity may be specific for organ systems, tissues, and/or epithelia [1, 31].

Currently, little evidence supports the formation of DNA damage directly by bile acids. The information is controversial. Some authors state the impossibility of formation bile acid-related DNA adduct in vitro [33, 34] whereas, by contrast, others report that bile acids or conjugates of glycocholic acid (**1**) and taurochenodeoxycholate sodium (**2**) are responsible for the elevation of DNA damage on esophageal cell lines [20, 35]. Moreover, bile acids and the oxidized forms, oxysterols, are linked to development and progression of cancers of the pancreas, colon, lung and breast [3, 36, 37]. With respect to helminthic infections, opisthorchiasis is associated with an elevation of bile acids, including deoxycholic acid, which is a potent promoter in cholangiocarcinogenesis [7]. Indeed, several metabolites and DNA adducts detected in this study have been implicated during schistosomiasis and opisthorchiasis [5–8].

Helminth infections caused by *Opisthorchis* species and *S. haematobium* are directly linked to malignancy [2]. We have shown that metabolites excreted by parasites might act as chemical carcinogens and act as initiators of carcinogenesis, through interaction with host DNA [3, 4, 38]. Here, we demonstrated the in vitro generation of several metabolites of glycocholic acid (**1**) and taurochenodeoxycholate sodium (**2**), precursors of DNA-adducts related to opisthorchiasis and urogenital schistosomiasis [5, 6, 8]. Also, we confirmed the ability of these metabolites to interact with DNA leading to the formation of DNA-adducts. The activation and subsequent formation of DNA-adducts seem to be performed through a CYP450-independent pathway, at least CYP1A1, CYP1B1, and CYP3A4. Some of the metabolites previously detected in the helminths themselves and infected people, and in a rodent model of infection, were synthesized in this study (Figs. 2, 3). Future studies using informative cell lines can be expected to define the carcinogenic roles of these metabolites.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13027-019-0257-2.

Additional file 1. Chromatograms obtained by LC-MS/MS of different aliquots analyzed.

Additional file 2. All m/z detected during analysis of LC-MS/MS of aliquots of samples and control during the course of reaction. Common and exclusive m/z detected for each of aliquots.

Additional file 3. Postulated structures for common compounds to different aliquots.

Additional file 4. Postulated structures for exclusive m/z detected on samples and control during course of reaction.

Abbreviations

+CYP: sample; *ACN*: acetonitrile; *CCA*: cholangiocarcinoma; *-CYP*: control; CYP450: *cytochrome P450*; *DMSO*: dimethylsulfoxide; *DNA*: deoxyribonucleic acid; *ESI*: electrospray ionization; *EtOH*: ethanol; *HF*: formic acid; *HPLC-MS/MS*: high performance liquid chromatographic mass spectrometer; *m/z*, ratio mass/charge; *IARC*: International Agency for Research on Cancer; *NADPH*: nicotinamide adenine dinucleotide phosphate; *PDA*: photo diode assay; *SCC*: squamous cell carcinoma; *UGS*: urogenital schistosomiasis; *UV*: ultraviolet

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Authors contribution

Conceptualization, N.V. and M.J.G.; Methodology, M.J.G.; Writing - Original Draft Preparation, M.J.G. and N.V.; Writing - Review & Editing, N.V., G.R., P.B.; Supervision, N.V., F.G., J.M.C.C.; Project Administration, N.V. and J.M.C.C.; All

authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflicts of interest.

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Chapter 4

The antioxidants resveratrol and N-acetylcysteine enhance anthelmintic activity of praziquantel and artesunate against Schistosoma mansoni.

CHAPTER 4. The antioxidants resveratrol and *N*-acetylcysteine enhance anthelmintic activity of praziquantel and artesunate against *Schistosoma mansoni*.

Authors: Maria João Gouveia, Paul J. Brindley, Carlos Azevedo, Fátima Gärtner, José Manuel Correia da Costa, Nuno Vale

The main purpose of this research work was evaluated the efficacy our novel therapeutic approach against newly transformed schistosomula (NTS). We used a host-parasite model involving *Bioamphalaria glabrata* and *Schistosoma mansoni* to established mechanical transformation of *S. mansoni* cercariae into NTS and optimized their culture conditions. Thereafter, we investigate the antischistosomal activity and ability of antioxidants as *N*-acetylcysteine (NAC) and resveratrol (Resv) to enhance the performance of praziquantel (PZQ) and/or artesunate (AS) against larval stage of the parasite. The efficacy of drugs either alone or combined was evaluated either by using an automated microscopical system and by transmission electron microscopy (TEM). The results obtained demonstrated that combination of PZQ+Resv and AS+Resv displayed synergy. In consonance to these results, TEM observations demonstrated that combination of AS+Resv induced severe, extensive alterations to the tegument and subtegument of NTS when compared to the compounds alone. The synergies observed might be the consequence of increased action by Resv on targets of AS and PZQ and/or they might act concomitantly on discrete targets to enhance overall antischistosomal action.

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The antioxidants resveratrol and *N*-acetylcysteine enhance anthelmintic activity of praziquantel and artesunate against *Schistosoma mansoni*

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Abstract

Background: Treatment of schistosomiasis has relied on the anthelmintic drug praziquantel (PZQ) for more than a generation. Despite its celebrated performance for treatment and control of schistosomiasis and other platyhelminth infections, praziquantel has some shortcomings and the inability of this drug to counteract disease sequelae prompts the need for novel therapeutic strategies.

Methods: Using a host-parasite model involving *Biomphalaria glabrata* and *Schistosoma mansoni* we established mechanical transformation of *S. mansoni* cercariae into newly transformed schistosomula (NTS) and characterized optimal culture conditions. Thereafter, we investigated the antischistosomal activity and ability of the antioxidants *N*-acetylcysteine (NAC) and resveratrol (RESV) to augment the performance of praziquantel and/or artesunate (AS) against larval stages of the parasite. Drug effects were evaluated by using an automated microscopical system to study live and fixed parasites and by transmission electron microscopy (TEM).

Results: Transformation rates of cercariae to schistosomula reached ~ 70% when the manipulation process was optimized. Several culture media were tested, with M199 supplemented with HEPES found to be suitable for *S. mansoni* NTS. Among the antioxidants studied, RESV alone or combined with anthelminthic drugs achieved better results rather *N*-acetylcysteine (NAC). TEM observations demonstrated that the combination of AS + RESV induced severe, extensive alterations to the tegument and subtegument of NTS when compared to the constituent compounds alone. Two anthelmintic–antioxidant combinations, praziquantel-resveratrol [combination index (CI) = 0.74] and artesunate-resveratrol (CI = 0.34) displayed moderate and strong synergy, respectively.

Conclusions: The use of viability markers including staining with propidium iodide increased the accuracy of drug screening assays against *S. mansoni* NTS. The synergies observed might be the consequence of increased action by RESV on targets of AS and PZQ and/or they may act through concomitantly on discrete targets to enhance overall antischistosomal action. Combinations of active agents, preferably with discrete modes of action including activity against developmental stages and/or the potential to ameliorate infection-associated pathology, might be pursued in order to identify novel therapeutic interventions.

Keywords: Anthelmintic, Resveratrol, *N*-acetylcysteine, Biomolecules, Schistosomula, *Schistosoma mansoni*

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Background

Schistosomiasis is considered the most important helminthic disease of humanity in terms of morbidity and mortality; more than 240 million people are infected with schistosomes, and another 700 million are at risk of infection [1–4]. Despite the fact that control strategies have been employed to block transmission and reduce the disease burden, including mass and targeted chemotherapy, improvements to sanitation and modification of the environment, and the use of molluscides [5], schistosomiasis remains a major public health problem in sub-Saharan Africa [3, 6]. Historically considered restricted to the tropics and subtropics where suitable intermediate host snails also are endemic, transmission of schistosomiasis has recently re-emerged in southern Europe [7]. Currently, chemotherapy is the first-line tool to minimize the prevalence and incidence of schistosomiasis [8–10]. Indeed, for the past 40 years praziquantel (PZQ) has been recommended by the World Health Organization for the treatment of all forms of schistosomiasis [11]. PZQ is inexpensive, given by mouth, readily available and well-tolerated, and hence suitable for mass drug treatment campaigns [11]. However, whereas PZQ is active against the adult developmental stages and against young schistosomula within a day or so of infection, it displays poor efficacy against schistosome eggs and the developing and migrating immature schistosomula and young adult forms [12]. This likely explains and contributes to low cure rates and rapid re-infection where residents of endemic sites are frequently infected with both juvenile and adult parasites concurrently. For effective treatment and sustainable control, PZQ retreatment must be maintained on a regular basis.

On the other hand, the dependence on PZQ raises legitimate concerns about the appearance of drug resistance [12–14]. Although widespread resistance has not been convincingly demonstrated, field and experimental isolates displaying reduced sensitivity to PZQ have been described from several countries [15]. The discovery and development of novel effective drugs, and also new drug targets, has been considered a research priority. PZQ derivatives do not improve antischistosomal activity. Frequently, the promising *in vitro* activity of a candidate PZQ-derivative has not been translated into antischistosomal activity *in vivo* [10]. There is a reawakening of the need and value to search for alternative chemotherapeutic tactics, such as combinations of drugs and drug repurposing [16–18].

PZQ alone cannot capably reverse pathological sequelae of schistosomiasis [19], and new therapies against schistosomiasis should focus also on this problem in addition to anthelmintic performance. During the past 30 years, artemisinin derivatives, such as artesunate (AS), have been shown to have antischistosomal activity both *in vitro* and in animal models. In marked contrast to PZQ, AS exhibits potent activity against juveniles whereas the invasive stages and adult worm are less susceptible. Moreover, adult female worms are somewhat more susceptible than the males. Although the mechanism of action of AS against schistosomes is not well understood, the glycogen content of worms is reduced by a reduction in glucose uptake, an increase in glycogen phosphorylase activity, and by inhibition of enzymes involved in glucose metabolism [20, 21].

During schistosomiasis, alterations occur in organs and tissues including disturbance to cellular antioxidant systems, which likely degrade the detoxification process of exogenous or endogenous free radical liberation, e.g. reactive oxygen species (ROS), which originate during the immunological response [22, 23]. On the other hand, reactive electrophilic compounds, e.g. estrogen-like metabolites, capable of reaction with DNA to form DNA-adducts and liberation of ROS, have been implicated as initiators of squamous cell carcinoma during urogenital schistosomiasis caused by infection with *Schistosoma haematobium* [24]. In this situation, the need for antioxidants increases to counteract reactive xenobiotics arising from oxidation [25–27], and in support of immunological and inflammatory responses directed at schistosome eggs in tissues [26]. Moreover, antioxidants might prevent DNA damage [28] and block the initiation of carcinogenesis [29].

Antioxidants such as *N*-acetylcysteine (NAC) and resveratrol (RESV) might ameliorate hepatic redox homeostasis during schistosomiasis. In addition, their protective effects against liver fibrosis induced by granuloma formation during infection may account partially for the ability of these antioxidants to inhibit or ameliorate the formation of schistosomal toxic products and render their impact reversible [26, 30]. NAC is an acetyl derivative of L-cysteine containing a thiol group, which participates in known biochemical pathways including its role as a precursor of cysteine, which is the rate-limiting component of glutathione (GSH). Moreover, NAC itself serves as an antioxidant by reacting directly with free radicals [31]. RESV is a 3,4,5-trihydroxylstilbene, a naturally occurring polyphenol occurs in flowering plants where it plays a role in homeostasis during environmental stress [32]. RESV exhibits neuroprotective and cardio-protective benefits [33, 34]. Not only is RESV an antioxidant, it also induces others intracellular antioxidant activities [34].

It has been emphasized that more research should be undertaken to investigate whether combinations with active compounds would reveal synergistic effects that could contribute to enhanced anthelmintic outcomes [35]. Despite having the above-described positive

attributes, both and PZQ and AS present some drawbacks. We speculated that combinations of these anthelmintic drugs with antioxidant biomolecules might enhance the antischistosomal performance of PZQ and AS. We investigated the schistosomicidal activity *in vitro* of combinations of PZQ and AS with the antioxidants NAC and RESV against newly transformed schistosomula (NTS) of *S. mansoni*.

Methods

Drugs and media

PZQ, NAC, Medium 199, HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid) (1M), L-glutamine, penicillin and streptomycin, Hank's balanced salt solution (HBSS), and amphotericin B were purchased from Sigma-Aldrich (Lisboa, Portugal); heat inactivated fetal bovine serum (iFBS) from Lonza (Basel, Switzerland); RESV from Santa Cruz Biotechnology (Dallas, TX, USA); and AS from Bertin Pharma (Montigny-le-Bretonneux, France). For *in vitro* assays, stock solutions of test compounds (2–4 mg/ml) were prepared in 100% dimethylsulfoxide (DMSO; Sigma-Aldrich) and stored at 4 °C.

Transformation of cercariae into newly transformed schistosomula (NTS)

To evaluate the schistosomicidal activity of antioxidants and whether or not they might augment the activity of anthelmintic drugs, cercariae of *S. mansoni* were mechanically transformed into schistosomula by vortexing transformation. NTS were obtained by mechanical transformation of *S. mansoni* cercariae shed from *Biomphalaria glabrata* for 2–3 h after exposure to light, with mechanical transformation performed as described [36]. Some parameters including duration of cercarial suspension on ice (30 to 60 min), centrifugation time (5 to 10 min), centrifugal force (800 to 1000× *rpm*) and steps during purification (3 to 5) were modified to enhance conversion rates of cercariae into schistosomula. The final step consisted of chilling the cercarial suspension on ice for 60 min, after which cercariae were pelleted by centrifugation at 1000× *rpm* at 4 °C for 10 min. The cercarial pellet was resuspended in 2 ml of cold HBSS containing 2% amphotericin B, mixed vigorously through a pipette, vortexed for 4 min to induce tail shedding, and incubated on ice for 10 min to concentrate the NTS. The tail-rich supernatant was decanted and discarded, and the pelleted schistosomula re-suspended in 7 ml of cold HBSS, with this step repeated five times. The conversion rate was calculated by counting the total number of cercariae before transformation in relation to the total number of schistosomula obtained after purification (Table 1).

Optimizing the culture conditions

After transformation of cercariae into NTS, optimal culture conditions were established iteratively. Due to microbial contamination from the schistosome-infected snails and snail excrement, culture media for NTS were supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin [37]. NTS were incubated in supplemented media M199 at 37 °C in 5% CO_2 in air [37]. NTS were placed in 96-well flat-bottom plates (Nunclon, Roskilde, Denmark) and incubated with M199 supplemented with 20 mM HEPES or 7.5% sodium bicarbonate and increasing concentrations of heat inactivated fetal bovine serum (iFBS), 5–15%, 37 °C in 5% CO_2 in air. The viability of NTS, at 50–100 larvae per well, was assessed daily based on morphology and motility using a semi-quantitative grading, where a score of 3 indicated normal activity without morphological changes; 2 indicated activity with some morphological changes and/or granularity; 1 indicated minimal activity, severe morphological changes and granularity; and 0 indicated no movement seen, severe granularity, non-viable [37–39] as observed under bright field at 10–40× magnification with an inverted microscope (Nikon Phase Contrast 2, LDW 0.52; Nikon, Tokyo, Japan). Schistosomula were considered to have died when movement was not evident after 90 s [38]. Micrographs were captured using a camera (PowerShot A360; Canon, Maryland, USA).

Antischistosomal activity

A concentration of 50–100 NTS per 100 µl in pre-heated (37 °C) optimal culture media was placed in 96-well flat-bottom plates (Nunclon) and incubated for 24 h at 37 °C in 5% CO_2 in air [38]. Culture media, 250 µl final volume per well, were supplemented with test compounds at increasing concentrations. NTS incubated in medium containing the maximum DMSO concentration, 2% v/v, served as the vehicle control. In a first screening, NTS were incubated for 72 h at the highest concentration (100 µM) of PZQ, AS, NAC and RESV alone and combined (e.g. PZQ-NAC) 1:1 (v/v). Secondary screening was performed iteratively based on antioxidant concentration performance during the initial screen. At the second screen, serial dilutions from 10 to 100 µM were tested. Initially, viability and morphological alterations 1, 17, 24 and 48 h post-exposure were assessed using inverted microscopy. After 72 h, NTS viability was assessed with the assistance of automated microscopy (LionHeart FX, BioTek, Winooski, VT, USA) fitted with Gen5 v.3.0 software to process and analyze data, to capture color bright field and fluorescence Texas Red channel (586 nm) images. Propidium iodide [PI; 0.5 mg/ml in sodium citrate (1%)] was added to each well and NTS incubated for

Table 1 Conversion rates for newly transformed schistosomula (NTS) *of Schistosoma mansoni* obtained using a modified vortex transformation procedure

Experiment	Description	Cercarial suspension (ml)	No. of cercariae	No. of schistosomula	Conversion rate (%)	Mean ± SD	Observations
1	Reduced motility: 30 min on ice; centrifugation: 800× *rpm*, 5 min, 4 °C; purification: 3 steps (15 min on ice)	35	3500	1750	50.0	[a]	Experiment 1 and 2: lower conversion rate; considerable number of tails and cercariae detected
2	Reduced motility: 30 min on ice; centrifugation: 800× *rpm*, 5 min, 4 °C; purification: 5 steps (15 min on ice)	38	6080	2800	47.0	61.4 ± 10.2	
3		22.5	3825	2900	75.8		Despite an increase in the schistosomula conversion rate, several tails were observed in wells
4	Reduced time between purification steps (15–10 min) (centrifuged 2× in order to obtain a solid pellet); slight increase of vortexing time	45	5850	3240	55.0	[a]	Alteration of culture supplemented media M199, 20 mM HEPES, 10% iFBS. It is probable that the time of initial suspension on ice was insufficient to decrease parasite motility, which led to a loss of cercariae after supernatant removal
5		25	10,000	–	–		Due to higher number of cercariae in initial suspension, it is probable that the time on ice was not enough to reduce motility. Consequently, it did not form a solid pellet after centrifugation leading to release of cercariae after removal of supernatant
6	Experiments 6–12: reduced motility: 60 min on ice; centrifugation: 1000× *rpm*, 10 min, 4 °C; purification similar to experiments 4 and 5; introducing check-points during the transformation method	50	5250	3720	70.9	68.6 ± 4.8	Fewer cercariae and tails detected. Good parasite fitness. Increased of concentration of iFBS to 15%. Final culture media established as supplemented M199 with HEPES and 10% FBS
7		100	11,750	7380	62.8		Due to a great number of cercariae, mechanical transformation was performed on separated tubes
8		40	12,000	8640	72.0		Experiment 8 and 9: lower number of tails and cercariae. Good parasite fitness
9		50	3000	2250	75.0		
10		27.5	1640	1040	63.4		Lower number of cercariae because several infected snails had died, thus only a few snails were infecting and shedding cercariae. In this case, we only performed 4 steps for purification in order to reduce loss of schistosomula during the process
11		50	4625	3000	64.8		Lower number of tails and cercariae, as well good parasite fitness
12		50	7375	5265	71.4		

[a] Unable to calculate mean and standard deviation (SD)

15 min a 37 °C [40, 41]. The principle is based on the different membrane permeability to the membrane-impermeable fluorescent DNA intercalating agent PI which stains membrane-compromised cells (red fluorescence) [40]. The performance of each combination was characterized using a combination index (CI), where CI > 0.1 indicates very strong synergism, CI: 0.1–0.3 strong synergism, CI: 0.3–0.7 synergism, CI: 0.7–0.85 moderate synergism, CI: 0.85–0.9 slight synergism, CI: 0.9–1.1 nearly additive and CI > 1.1 antagonist, as previously described [42, 43]. This was calculated with CompuSyn v.1.0 (ComboSyn, Inc., Paramus, NJ, USA). Each concentration of anthelmintic drug, antioxidant alone and in combination was tested in duplicate; the assays were performed at least twice.

Transmission electron microscopy (TEM)

For evaluation of ultrastructural alterations induced by AS, RESV or AS + RESV (1:1) at 100 μM and post-exposure of 72 h, the NTS treated and untreated (controls) were fixed with 2.5% glutaraldehyde in 0.2 M sodium cacodylate, pH 7.4 for 4 h. After fixation, the worms were washed overnight in the same buffer and post-fixed in 1% osmium tetraoxide (OsO_4). Subsequently, fixed NTS were dehydrated in an ascending, graded ethanol series and embedded in Epon epoxy resin. Semi-thin sections were stained with methylene blue-Azur II. Ultrathin sections were double-contrasted with aqueous uranyl acetate and lead citrate. Ultrastructural features of NTS in the sections were observed and images documented with TEM using a JEOL 100CXII microscope (JEOL, Massachusetts, USA) operated at 60 kV and equipped with a Gatan digital camera (Gatan, California, USA).

Results

Mechanical transformation

The mechanical transformation of cercariae of *S. mansoni* into NTS using the revised protocol yielded an average conversion rate of 68.6 ± 4.8% (mean ± SE) compared to 47% using an earlier method [36], as well as fewer cercarial tails in the culture plates during the downstream assays (Table 1, 12 experiments). Low conversion rates seen in Experiments 1 and 2 likely were related to the shorter time on ice; hence, the larvae were more mobile. Centrifugation at 800× *rpm* for 5 min might be insufficient to pellet the larvae, so that cercariae may be inadvertently discarded with the supernatant. During Experiment 3, the conversion rate was higher than in Experiments 2 and 4 which may be related to the lower number of cercariae in the initial suspension. Thus, the time on ice might be sufficient to reduce motility and/or the reduction of the volume of initial suspension may favor a fast sedimentation and formation of solid pellet of cercariae after centrifugation. During the mechanical transformation, it is necessary to consider additional parameters that may vary from assay to assay, including numbers of infected snails, numbers of cercariae, and volume of cercarial suspension. We observed that steps needed to be adjusted during transformation in order to improve the conversion rates. For example, if the initial number of cercariae in suspension was higher (~ 10,000 per 50 ml), more time on ice to reduce parasite motility and more vortexing time to induce removal of the tail were both required. However, it is necessary to caution that increased vortexing can injure the larvae [37]. Several checkpoints were performed in order to assess parasite fitness and tail-loss during transformation. It should be noted that the number of cercariae obtained in initial suspensions was variable (Table 1) since it varied between snails, and was dependent upon additional factors including number of infected snails and number of times the cercariae had been shed from the snails.

Optimal culture conditions for *S. mansoni* NTS

NTS cultured in medium M199, supplemented with 7.5% sodium bicarbonate and 5% iFBS, died after 72 h. Parasites incubated in M199 supplemented with 20 mM HEPES and 10% iFBS, remained viable for at least 96 h without membrane disruption and/or marked morphological changes (Additional file 1: Figure S1). These NTS displayed an average viability value of about 2.5 (not shown). Increasing the concentration of iFBS to 15% did not enhance viability. Therefore, M199 supplemented with 20 mM HEPES and 10% iFBS appeared to be suitable for incubation of NTS by vortex transformation and was used for the drug sensitivity assays.

In vitro S. mansoni NTS drug sensitivity assay

Antischistosomal activity of antioxidants (NAC and RESV) and their ability to enhance antiparasitic activity of anthelmintic drugs (PZQ and AS) was assessed on NTS derived from successful mechanical transformation. As previously described [38], in the absence of test compounds, NTS showed normal viability without any morphological changes for up to 24 h. Mild changes in granularity and motility were apparent following 48 h of incubation. NTS remained viable for at least 96 h [38]. Generally, incubation in PZQ and AS at the highest concentration (100 μM) caused severe deformity and granularity scores of 1 (slow activity and severe granulation) on the viability scale of NTS (Additional file 1: Figures S2 and S3). However, none of the drugs alone were capable of inducing the death of all NTS (Additional file 1: Table S1). Following 24 h in AS, NTS did not show significant morphological alterations in comparison to controls. However, after 48 h the larvae were granular in appearance,

some markedly, irregular in shape, and movement was reduced. By 72 h, most NTS were dead (Additional file 1: Table S1). PZQ strongly decreased viability but did not cause the death of NTS. Initially, PZQ elicited an overactive phenotype progressing to overactive/degenerate but motile, i.e. NTS were motile yet severely disrupted in morphology. In addition, we observed spheroid-shaped worms (rather than vermiform), which were markedly granulated, as described by other investigators [44, 45]. Although PZQ inflicted more damage, AS was more active than PZQ inducing more mortality among NTS (Additional file 1: Table S1), consistent with other reports that indicated that AS was more effective against larval and young stages of *S. mansoni* [20]. The antioxidants NAC and RESV were tested at up to 100 µM for anthelmintic activity. Neither NAC nor RESV killed NTS after 72 h of incubation (Additional file 1: Figure S2). Nevertheless, the percentage of dead NTS induced by the Resv compound was higher rather than NAC (Additional file 1: Table S1). NAC induced only slight morphological alterations, specifically an increase in granularity at the highest concentration tested (Additional file 1: Figure S3). RESV induced an increase in granularity, and reduced motility (Additional file 1: Figure S3). To investigate synergism between PZQ or AS and the antioxidants, NTS were incubated with a constant dose ratio (1:1) of the highest concentration (100 µM) of a combination of each anthelmintic and antioxidant (Additional file 1: Table S1). Combinations of PZQ or AS with NAC achieved the same viability score and similar percentage of death as the anthelmintic drug alone (Additional file 1: Figures S2, S3 and Table S1). The morphological alterations and mortality of NTS incubated with PZQ + NAC were generally identical with those induced by drug alone (Additional file 1: Table S1 and Figure S5). The NTS exhibited less granularity and rounded shape than seen in larvae incubated in PZQ alone. Similarly, incubation with AS + NAC led to severe granularity, loss of motility and changes in shape identical with changes induced by AS alone (Additional file 1: Figures S2 and S3). Curiously, the number of dead NTS was lower in combination in comparison to the drug alone (Additional file 1: Table S1 and Figure S5). In contrast, combinations of PZQ or AS with RESV markedly enhanced the *in vitro* effects as compared with the anthelmintic alone. Indeed, the percentage of effects achieved by combinations was higher in comparison to compounds alone (Additional file 1: Table S1). Notably, only AS + RESV killed all NTS after 72 h, indicating that RESV enhanced the anthelmintic performance of AS (Additional file 1: Table S1 and Figure S5). Figure 1 depicts the temporal effect of AS + RESV at constant dose ratio (1:1) at the highest concentration used. Notably, RESV achieved better results than NAC in all combinations examined (data not shown).

Figure 2 illustrates the alterations in morphology induced by the anthelmintic drugs PZQ or AS and RESV alone as well as their combination at the highest concentration, after incubation for 72 h. Real time morphological alterations of the schistosomula were assessed with an inverted microscope and with the Biotek LionHeart FX automated imaging microscopy platform. Images demonstrating relevant differences among the morphology of NTS incubated with AS, PZQ and RESV, alone and in combinations, were captured. Despite several morphological alterations induced by 100 µM RESV, the NTS maintained membrane integrity (red arrow) and showed minimal activity while PZQ induced the rounded shape effect and severe granularity, although the NTS remained motile, as previously described [44, 45]. The combination of PZQ + RESV induced significant morphological alterations, notably blebbing (black arrow). In the assays with AS, the anthelmintic alone or the combination AS + RESV induced severe granularity,

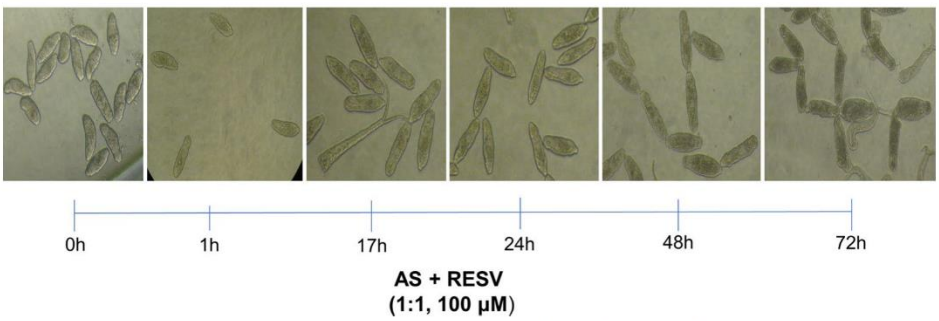

Fig. 1 Temporal effect of exposure of schistosomula of *Schistosoma mansoni* for 72 h to anthelmintic drugs and antioxidants (drugs AS + RESV 1:1, 100 µM)

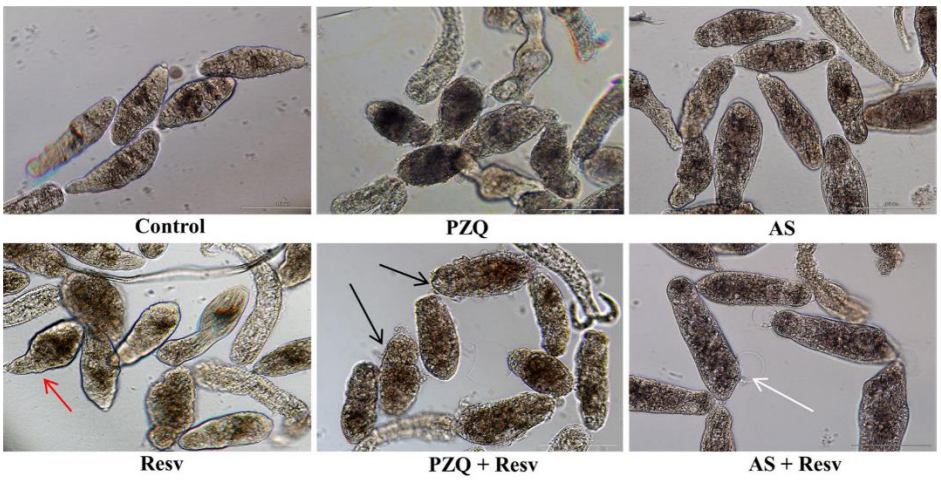

Fig. 2 Morphological alterations manifested by schistosomula of *S. mansoni* following exposure to anthelmintic drugs and RESV and their combinations. Newly transformed schistosomula were exposed for 72 h to PZQ, AS, RESV, PZQ + RESV and AS + RESV in a dose ratio of 1:1 at highest concentration (100 µM) and compared to controls. Generally, NTS showed dark granularity and alterations in shape that were more pronounced after exposure to combinations of anthelmintics and antioxidants that to anthelmintics or antioxidants alone. PZQ induced a round/oval shaped phenotype and severely disrupted morphology. Although RESV induced some morphological alterations, the tegmental integrity of NTS larvae remained intact (red arrow). PZQ + RESV induced not only severe granularity but also blebbing (black arrows). With AS + RESV, NTS showed membrane disruption (white arrow) followed by death. Images were captured using a BioTek LionHeart FX Automated Live Cell microscope (magnification of 20×)

alterations in shape and inactivated the NTS. Note that NTS incubated with AS + RESV suffered membrane disruption (white arrow), indicating the death of the schistosomulum.

Since RESV at maximum concentration (100 µM) showed antischistosomal effects alone and also potentiated the effects of the anthelmintic, combinations with lower concentrations of RESV were tested in the same (1:1) or in different concentration ratios in a secondary screen (Table 2). Combinations at a constant ratio (1:1) at different concentrations (10–100 µM) achieved better activity than the other constant ratios tested. As depicted in Table 2, all combinations induced higher NTS mortality compared to compounds alone (see also Additional file 1: Figure S5). At lower concentrations (< 100 µM) of anthelmintic and RESV (alone) or combined, only low to moderate activity was seen (Table 2). None killed all the NTS by 72 h and morphological alterations were less obvious. Accordingly, the highest percentage of dead NTS, about 30%, was induced by RESV at higher concentration (Table 2). However, similar to what was observed above, following incubation in 10–100 µM PZQ for 1 h, the NTS were overactive and showed visible granularity while those incubated for 72 h showed severe damage, although the worms remained motile. Despite the severe damage induced by PZQ, about 50% of NTS remained alive (Table 2). Nonetheless, NTS incubated with the combined anthelmintic and RESV showed more morphological alterations at all concentrations, in terms of granularity, minimal movement, and alteration in shape, in comparison to NTS incubated with the same compounds alone (Additional file 1: Figure S4).

In addition to the bright-field microscopical-based assessment, NTS viability was also assessed incorporating a red-fluorescent dye that objectively detects parasite survival during *in vitro* culture. Following 72 h, dead

Table 2 Percentage (mean ± SD) of dead NTS induced by compounds alone and its combinations for different concentrations obtained by staining with iodide propidium

Concentration (µM)	Control	PZQ	AS	RESV	PZQ + RESV	AS + RESV
10	0.38 ± 0.18	45.7 ± 2.1	18.0 ± 3.5	12.3 ± 2.0	38.2 ± 3.7	23.7 ± 4.0
50	1.50 ± 0.71	52.7 ± 0.7	37.0 ± 3.4	28.1 ± 3.1	69.2 ± 2.8	35.5 ± 5.4
100	1.38 ± 0.88	56.9 ± 2.5	70.0 ± 3.8	30.0 ± 1.6	81.0 ± 5.2	99.9 ± 0.1

NTS were stained with PI and the plate examined was readout using the Texas Red filter at 586 nm on a BioTek LionHeart Automated Live Cell microscope (Fig. 3). As expected, all NTS incubated with AS-RESV were stained and were even brighter than others indicating that they were dead (Table 2). It is noteworthy that the number of dead schistosomula was lowest in control, followed by RESV, PZQ, AS and PZQ + RESV (Table 2). All combinations of anthelmintic drugs with RESV yielded synergistic antischistosomal effects. Based on these findings, we conclude that the combination of AS + RESV was identified as synergistic (CI = 0.34), near to strong synergism; and moderate synergism was observed for PZQ and RESV (CI = 0.74). These findings conformed with the microscopical observations: the combination of AS with RESV was more active against *S. mansoni* NTS *in vitro* than PZQ + RESV (Table 2, Fig. 4).

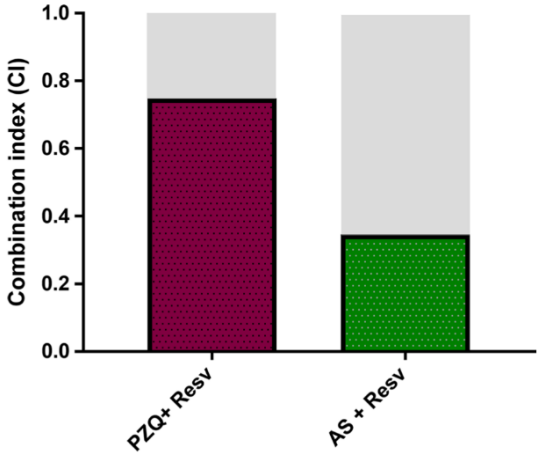

Fig. 4 Combination indices (CI) obtained for combinations of anthelmintic drugs and RESV against newly transformed schistosomula of *S. mansoni*. The combination of AS + RESV was synergistically active against the schistosomula. High synergism is indicated in gray

Transmission electron micrographs revealed that combination induces significative internal damage

TEM analysis was employed to investigate and compare ultrastructural damages induced by AS, RESV alone or in combination (AS + RESV) (Fig. 5). The ultrastructural features of NTS in the control group remained normal and were similar to that in reported earlier [46]. The analysis of NTS treated with AS, RESV alone or combined (AS + RESV) at 100 µM revealed marked alteration of ultrastructural features of tegument and subtegumental structures (Fig. 5). Following exposure to RESV, vesicles were present on the tegument and there was irregularity in the appearance of the membrane. TEM evaluation of NTS treated with AS revealed the loss of matrix integrity diffusely through subtegumental regions and the presence of vacuoles on the interior of organelles, which probably resulted from cytoplasmic processes. Nonetheless, there was no evidence of significant alterations of the tegument. By contrast, for NTS treated with AS + RESV, tegumental disruption and disappearance of basal membrane were apparent. In addition, lysis of the tegumental matrix was usually revealed close to the basal membrane leading to the formation of large vacuoles above the

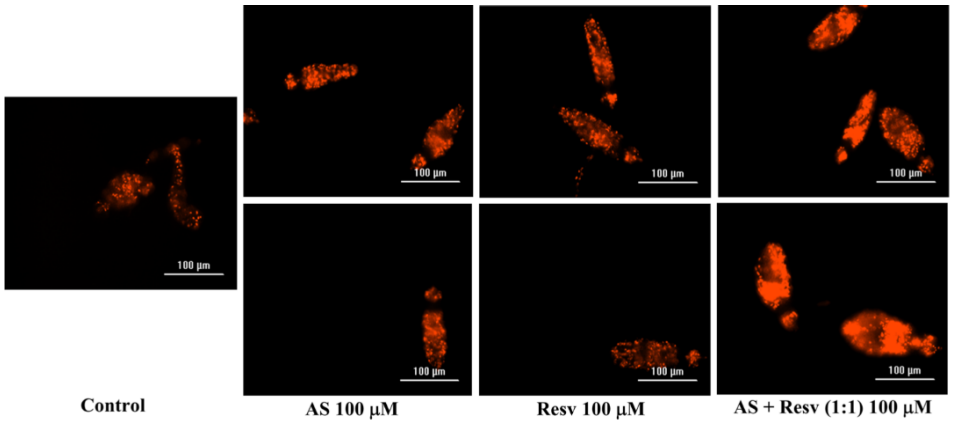

Fig. 3 *In vitro* effects of AS, RESV and AS + RESV (highest concentration) on viability of *Schistosoma mansoni* NTS assessed by propidium iodide (PI) incorporation following exposure for 72 h. Notably, NTS exposed to AS + RESV showed stronger fluorescence than schistosomula cultured in AS or RESV alone, indicating that the former was dead. Images were captured using the BioTek LionHeart FX Automated Live Cell microscope (magnification of 20×) fitted with a 586 nm (Texas Red) filter. *Scale-bars*: 100 µm

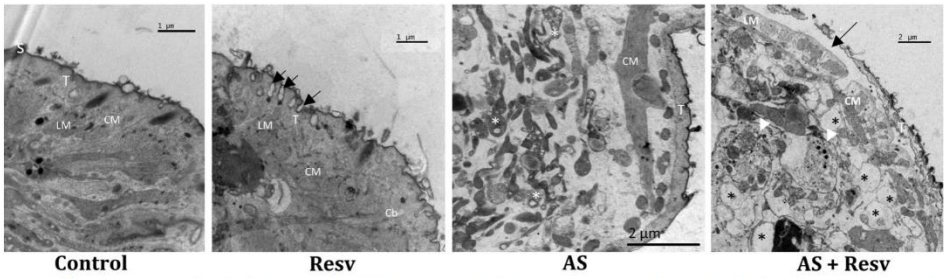

Fig. 5 Ultrastructural level micrographs of schistosomula of *S. mansoni* at 72 h after exposure to AS, to RESV and to the combination of AS + RESV. In the control group, schistosomula exhibits intact tegument (T), spines (S), circular and longitudinal muscle (CM, LM) with a regular morphology. For NTS exposed to RESV, vesicles were seen in the tegument (arrows) along with some disorganization; no apparent damage occurred in subtegumental regions. In contrast, for schistosomula exposed to AS, loss of matrix integrity but without alterations to the tegument was apparent. The most prominent damage was seen on NTS treated with the combination of AS + RESV. Here, NTS displayed disruption and lysis of internal structures (arrowheads) and swelling of parenchyma tissues, disruption of tegument and disappearance of basal membrane (arrow), and the appearance of large vacuoles (asterisks). *Abbreviations*: T, tegument; S, spine; LM, longitudinal muscle; CM, circular muscle; Cb, cytoplasmatic bridge. *Scale-bars*: 1 µm (Control and Resv); 2 µm (AS and AS + Resv)

basal membrane. The subsequent dysfunction or leakage of internal contents might be involved upon cytoplasmic lysis. It seems likely that disruption of the tegument might be directly linked to death of NTS treated with AS + RESV. Upon comparison of the ultrastructure of NTS treated with these compounds alone or in combination, it was possible observe that the damage was more prominent following combination treatment, reinforcing the notion that that RESV enhanced the antischistosomal activity of AS. The TEM micrographs were consistent with the findings obtained by light microscopy where it was possible to observe the presence of membrane disruption in the NTS treated with AS + RESV and extensive morphological alterations in contrast to the effects of AS or RESV alone (Fig. 2).

Discussion

Schistosomiasis is a major public health and economic burden in the tropical developing world [6, 47, 48]. Currently, there is one drug available as the core treatment of schistosomiasis, and it has been used extensively in mass administration for transmission control [49]. Problematically, emergence of PZQ-resistance is not unlikely, and indeed low cure rates with PZQ have been repeatedly reported [10, 15]. There is a pressing need for new therapeutic approaches that combine different modes of action and/or repurposing of drugs [17]. Combination chemotherapy is common in medicine, including treatment for cancer, bacterial infections, HIV and malaria, as well as in the veterinary and agricultural arenas [50, 51].

Here, after establishing tractable transformation and culture conditions for NTS, drug sensitivity screenings were undertaken using bright field, visual inspection with an inverted microscope and also by automated microscopy using a BioTek LionHeart system. AS was more active than PZQ on NTS, which is consistent with earlier findings that showed that AS is more effective against larval and immature mammalian stages of *S. mansoni* due to the presence of the endoperoxide bridge that induces the production of ROS. By contrast, PZQ is more active against adult schistosomes [20]. Alone, NAC and RESV demonstrated modest activity against NTS. Although NAC did not exhibit antischistosomal activity against NTS *in vitro*, it might ameliorate redox homeostasis and host morbidity by downregulating oxidative stress caused by infection [26]. RESV induced more marked morphological changes than NAC on schistosomula; perhaps RESV acted on neuromotor activity, based on its effects on motility, which in turn could degrade its ability to migrate and acquire nutrients. Indeed, in *S. mansoni* infected mice, the administration of RESV ameliorates oxidative stress and organ dysfunction [30]. These effects likely are not only related to biological properties of RESV but also host antischistosomal activity. RESV might promote a combined action by both harming the schistosome while also ameliorating host oxidative stress.

With respect to the combinations, NAC did not enhance the activity of AS or PZQ. By contrast, AS or PZQ in combination with RESV improved performance in terms of antischistosomal effect, more than the single compound. This finding indicated that RESV possesses and enhances antischistosomal activity of both these anthelmintics. These outcomes were evident following serial dilution of AS, PZQ and RESV (alone) and combined. At dilute concentration, augmented activity against NTS was evident. Indeed, combinations of PZQ or AS with RESV presented a moderate (CI = 0.74) and marked (CI = 0.34) synergistic effect, respectively.

Synergy in antischistosomal action might result from increasing the action on anthelmintic drugs targets or acting concomitantly on different targets [52].

The ultrastructural analysis demonstrated that NTS treated with AS + RESV suffered extensive and severe damage in comparison to controls and NTS treated with AS or RESV. The tegumental and subtegumental regions of these NTS showed alterations including disruption of tegument, extensive lysis of subtegumental regions with presence of numerous and vacuoles with diverse sizes, and loss of the basal membrane. Nonetheless, NTS treated with AS or PZQ alone also showed ultrastructural alterations, including loss of integrity of the matrix in the case of AS, and presence of vesicles on the tegument and some tegument disorder of larvae treated with RESV. With regard to controls, these presented regular morphology. Taken together, the light microscopic and TEM micrographs revealed that RESV not only induced alterations on the tegument of NTS but also augmented the antischistosomal activity of AS, leading to disruption of tegument and extensive lysis of subtegumental regions. The tegumental damage might lead might to disappearance of the immunological camouflage of the parasite which, in turn, would expose immunogens and immunogenic epitopes. These kinds of damage and immunological reactions represent a key process in the action of PZQ *in vivo* [53–56]. The schistosome tegument represents the frontline interface between host and parasite and plays a pivotal role in defence function to escape the host immune response. Additionally, it has essential secretory and nutrient absorption functions [57, 58]. Accordingly, tegumental disruption induced by AS + RESV would be anticipated to negatively affect the parasite's capacity to support its nutrition and to thwart host immune responses.

The assessment of parasite viability microscopically *in vitro* is based on regular lack of movement of larvae (motility) and morphological changes such as granularity and shape alterations [37] that might be subjective and inaccurate [41]. Accordingly, it is crucial to complement microscopic examination with fluorometric or staining approaches. We used a simple method based on the incorporation of PI for red fluorescence staining that does not require an extensive knowledge of schistosome biology [40]. A good correlation was observed between light and fluorescence microscopic readouts after exposing schistosomula to anthelmintics and RESV (alone) and combined, indicating that microscopic readout complemented with fluorometric methods represented an accurate and tractable technique to assess viability. Other methods to objectively quantify the activity of antischistosomal drugs also are available, including the xCELLigence approach [59, 60].

RESV and AS exhibited antischistosomal activity against schistosomula and synergism of antischistosomal effect was seen RESV or AS was combined with AS or PZQ. This synergistic effect was most pronounced with AS + RESV. Based on these findings, a combination of active agents, preferably with discrete modes of action including activity against developmental stages and to ameliorate infection associated pathology, might be pursued in order to identify novel therapeutic interventions. Investigation also should be undertaken to assess the synergies of these combinations against adult forms, other schistosome species, and schistosome infections in laboratory rodents. Indeed, we intend to evaluate these combinations in the *S. haematobium*-hamster model [24, 61] and for related trematodes responsible for hepatobiliary tract disease including cholangiocarcinoma [24, 62–66].

Conclusions

To conclude, RESV appeared to exhibit antischistosomal activity against schistosomula and also to induce synergism in combination with AS or PZQ. Based on these findings, we suggest that novel therapeutic interventions should be sought that involve the combination of active agents, preferably agents with discrete modes of action, and which also exhibit activity against developmental stages, and/or which also ameliorate infection-associated pathology.

Additional file

Additional file 1: Figure S1. Morphology of *S. mansoni* NTS after 96 h incubation using different supplemented M199. **Figure S2.** Viability score induced by drugs, antioxidants alone or combined. **Figure S3.** Morphological alterations induced by drugs (PZQ and AS), antioxidants (Resv and NAC) alone or combined. **Figure S4.** Morphological alterations observed at different concentrations of compounds. **Figure S5.** Graphical of percentage of dead NTS induced by compounds evaluated at different concentrations. **Table S1.** Percentage of dead NTS induced by compounds alone and combination evaluated at 100 µM and constant ratio 1:1.

Abbreviations

AS: artesunate; CI: combination index; DMSO: dimethylsulfoxide; iFBS: heat inactivated fetal bovine serum; NAC: N-acetylcysteine; NTS: newly transformed schistosomula; PI: propidium iodide; PZQ: praziquantel; RESV: resveratrol; TEM: transmission electron microscopy.

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Authors' contributions
NV, JMCC, FG planned and supervised the study. MJG performed the measurements and other assays. All authors processed the experimental data and drafted the manuscript including the figures. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article and its additional file.

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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Chapter 5

Combination anthelmintic/antioxidant activity against Schistosoma mansoni

CHAPTER 5. Combination anthelmintic/antioxidant activity against *Schistosoma mansoni*

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The research work presented in Chapter 5 consisted in a follow-up of that presented in previous chapter. In this study we used the same host-parasite model for *in vitro* drug screenings assays. Here, we selected different classes of drugs (anthelmintic and anticancer) and several antioxidants and evaluated either alone or combined. The morphological alterations induced by compounds alone or combined were assessed on daily basis using an inverted and automated microscope combine with a flurometric-based method to quantify NTS viability. The findings indicate that not only do some antioxidants improve antischistosomal activity of drugs, but also exhibited activity per se (e.g. Curc and 4-phenyl-1,2,5-oxadiazole-3-carbonile-2-oxide (OXA) and Curc) leading to high mortality of NTS post-exposure. The combination index (CI) for several combinations anthelmintic+antioxidant (e.g. PZQ+Mel, PZQ+Resv, AS+Resv, AS+NAC) and anticancer+antioxidant (e.g. VDT+Flavone (Flav), VDT+Resv) reveal that display moderate to strong synergism. Repurposing of drugs as anticancer (or others) seems to be worthwhile since they were affective against larval stage. We consider that this approach might be valuable for use in regions with intense re-infection levels since the combination might block or retard parasite infection and development.

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Combination Anthelmintic/Antioxidant Activity Against *Schistosoma Mansoni*

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Abstract: Schistosomiasis is a major neglected tropical disease. Treatment for schistosomiasis with praziquantel (PZQ), which is effective against the parasite, by itself is not capable to counteract infection-associated disease lesions including hepatic fibrosis. There is a pressing need for novel therapies. Due to their biological properties, antioxidant biomolecules might be useful in treating and reverting associated pathological sequelae. Here, we investigated a novel therapy approach based on a combination of anthelmintic drugs with antioxidant biomolecules. We used a host-parasite model involving *Bioamphalaria glabrata* and newly transformed schistosomula (NTS) of *Schistosoma mansoni*. For in vitro drug screening assays, was selected several antioxidants and evaluated not only antischistosomal activity but also ability to enhance activity of the anthelmintic drugs praziquantel (PZQ) and artesunate (AS). The morphological alterations induced by compounds alone/combined were assessed on daily basis using an inverted and automated microscope to quantify NTS viability by a fluorometric-based method. The findings indicated that not only do some antioxidants improve antischistosomal activity of the two anthelmintics, but they exhibit activity per se, leading to high mortality of NTS post-exposure. The combination index (CI) of PZQ + Mel (CI = 0.80), PZQ + Resv (CI = 0.74), AS + Resv (CI = 0.34), AS + NAC (CI = 0.89), VDT + Flav (CI = 1.03) and VDT + Resv (CI = 1.06) reveal that they display moderate to strong synergism. The combination of compounds with discrete mechanisms of action might provide a valuable adjunct to contribution for treatment of schistosomiasis-associated disease.

Keywords: *Schistosoma mansoni*; antioxidant biomolecules; anthelmintic drug; anticancer drug; drug repurposing; combination therapy; newly transformed schistosomula

1. Introduction

Since the 1970s, chemotherapy against schistosomiasis has relied on praziquantel (PZQ) which is considered the drug of choice for infection [1,2]. Despite PZQ efficacy against all forms of schistosome species presenting mild and transient side effects, it has major drawbacks as its poor efficacy against juvenile parasites and pharmacokinetic profile (e.g., extensive first-pass metabolism) [1,2]. In addition, massive and exclusive reliance on a single drug raises legitimate concern about schistosome PZQ-resistance. In fact, studies have reported field and experimental isolates that exhibit significantly reduced susceptibility, which might be a foreshadowing for emergence of resistance [3,4]. Moreover, PZQ-based treatment alone cannot reverse pathological sequelae of infection as periportal fibrosis for intestinal schistosomiasis and bladder deformity and hydronephrosis for urogenital schistosomiasis [5,6]. Taken together these considerations, there is a pressing need to search for additional therapeutics. Drug repurposing is an efficient approach to reduce time and cost of drug research and development [7,8], and/or combination of discrete biological active agents [9]. From our perspective, novel therapeutic approaches should focus not only on antischistosomal performance but also in amelioration of disease sequelae. During schistosomiasis, oxidative processes are triggered by liberation of reactive oxygen species (ROS) resulting from the immunological response and disturbance in cellular antioxidant homeostasis of affected organs [10,11]. We have suggested that reactive electrophilic compounds, e.g., estrogen-like metabolites of parasite origin initiate the carcinogenesis process associated with the chronic infection with *Schistosoma haematobium*. These metabolites are capable of interacting with host DNA leading to formation of DNA-adducts (releasing ROS) triggering a cascade of events that may lead to squamous cell carcinoma (SCC) of bladder associated to infection [12,13].

Due to the physiological properties of antioxidants, which are considered pharmacologically safe agents with minimal side effects [14,15] we speculated that combining these agents with anthelmintic drugs might offer enhanced antischistosomal action in combination with PZQ or other anti-worm drugs. Antioxidants have demonstrated antischistosomal activities [16], exhibiting differential performance among developmental stages of the parasite [17–21]. Moreover, the antioxidants resveratrol (Resv) and *N*-acetylcysteine (NAC) have demonstrable ability to prevent DNA damage [21] and block carcinogenesis [22–25].

Based on findings with drug repurposing and combinations of multiple biological active agent strategies, we report a novel therapeutic approach that combined anthelmintic drugs and anticancer with antioxidants biomolecules. We hypothesize that combinations of these active agents would reveal synergies that could be translated into enhanced antischistosomal activity. We demonstrated that not only do some antioxidants alone exhibit antischistosomal activity inducing severe morphological alterations and death of parasite but that they improve activity of established drugs including PZQ and artesunate (AS). AS is an antimalarial that unlike PZQ is effective against the juvenile forms of the schistosome [7,16]. In addition, we have also assessed the combination of anticancer drugs combined with antioxidant biomolecules. It has been demonstrated that kinase inhibitors as imatinib (IMT) interfere with essential development steps in the biology of schistosomes and causes degenerative changes in the gonads and gastrodermis [26–28]. Therefore, we included these agents here and evaluated antischistosomal activity in the presence of antioxidant biomolecules. The findings presented below reinforced the notion that combining biological agents with discrete modes of action might provide a valuable approach for treatment of schistosomiasis.

2. Materials and Methods

2.1. Chemicals and Culture Media

Praziquantel (PZQ), 4-phenyl-1,2,5-oxadiazole-3-carbonile-2-oxide (OXA), *N*-acetylcysteine (NAC), flavona (Flav), flubendazole (FBZ), and propidium iodide (PI) were purchased from Sigma-Aldrich (Lisboa, Portugal), and resveratrol (Resv) from Santa Cruz Biotechnology

(Heidelberg, Germany), artesunate (AS), vandetanib (VDT), curcumin (Curc), kaempferol (kaempf), and melatonin (Mel) from Cayman Chemical (Ann Arbor, MI, USA) and the dipeptide H-L-tryptophan-L-serine-OH (H-Trp-Ser-OH, DiPept) from Bachem (Bubendorf, Switzerland). The culture media M199, Hank's Balanced Salt Solution (HBSS) and supplements as HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (1M), penicillin (10,000 U/mL)/streptomycin (10 mg/mL), and amphotericin B were purchases from Sigma-Aldrich and heat inactivated fetal bovine serum (iFBS) from Lonza (Basel, Switzerland). For in vitro assays, stock solutions of (2–4 mg/mL) were freshly prepared in 100% dimethylsulfoxide (DMSO) (Sigma-Aldrich) and stored at 4 °C.

2.2. Parasites

Experimentally-infected *Bioamphalaria glabrata* snails maintained in water were exposed to artificial light during 2–3 h to shed cercariae of *Schistosoma mansoni* (isolate of Brazilian origin) maintained on Department of Infectious Diseases of National Institute of Health Dr. Ricardo Jorge (Porto). The number of cercariae in suspension was estimated and mechanically transformed into newly transformed schistosomula (NTS) as described [29,30] but with some modifications, as follows. The cercarial suspension was placed on ice to for 60 min to decrease motility of the larvae. Subsequently, the cercariae were pelleted by centrifugation and then resuspended in HBSS, 2% amphotericin B, vigorously stirred and slightly vortexed to induce tail loss tail. To recover the NTS, cold HBSS was added to the suspension and chilled on ice for 15 min. The supernatant now rich in tails was decanted and the pellet was resuspended in 7 mL cold HBSS. These steps were repeated at least twice. The conversion rate was calculated by counting the number of cercariae before transformation and the total NTS after enrichment. The NTS suspension was adjusted to a concentration of 50–100 NTS per 100 µL culture medium M199, 10% iFBS, 1% penicillin/streptomycin and dispensed into wells of a 96 well flat bottom plate (Nunclon, Roskilde, Denmark). Before addition of the compounds, the suspension was incubated at 37 °C, 5% CO_2 for 24 h to ensure complete conversion of cercariae to NTS.

2.3. In Vitro Drug Sensitivity Assay in NTS

The antischistosomal activity of anthelmintic, anticancer drugs and antioxidant biomolecules, either alone or combined (at ratio 1:1), was evaluated at concentration of 100 µM since it more suitable to test sublethal doses of natural product derivatives as antioxidants [31,32]. In combination, each drug and antioxidant were evaluated at 100 µM. To evaluate the effectiveness of novel therapeutic approach the 96 well flat bottom plates were prepared as follow: (1) 150 µL of pre heated (37 °C) supplemented M199 was placed in each well; (2) dilutions of compounds (alone or combined) were added to achieve final concentration of 100 µM, and, (3) NTS suspension was added to achieving final volume of 250 µL/well. The NTS containing the highest DMSO concentration (2% v/v) served as vehicle control. Initially, viability and morphological alteration at 1, 17, 24, and 48 h post-exposure to compounds were assessed using by inverted microscopy under bright field at 10–40× magnification (Nikon Phase Contrast 2, LDW 0.52, Tokyo, Japan) coupled with a camera (Canon PowerShot A360, Tokyo, Japan). To assess the phenotypic changes, we used a semi-quantitative viability scale as described below (Table 1).

Table 1. Semi-quantitative viability scale used to evaluate phenotypic changes of *Schistosoma mansoni* newly transformed schistosomula (NTS) induced by compounds alone and its combinations [8,33].

Viability Scale	Phenotypic Alteration
0	All worms dead; severe granularity
1	Minimal activity; severe morphological changes; granularity
2	Showed activity; first morphological change; granularity visible
3	Totally vital; normal activity; gross morphological changes not apparent

At 72 h post-exposure, an automated microscope LionHeart FX (BioTek, Winooski, VT, USA) incorporated with Gen5 3.0 software to process and analyze the data, was used in color bright field or fluorescence channel Texas Red (586 nm) to assessed NTS viability. In order to quantify the viability, a fluorophore (a solution of 0.5 mg/mL of PI) was added to the wells and incubated for 15 min at 37 °C [34]. Subsequently, the plates were read on the LionHeart FX microscope system using a fluorescence channel. The PI incorporate membrane-compromised cells staining with red fluorescence. Therefore, the parasites stained and with no movement for 90 s were considered to be dead [35]. The performance of each combination was characterized using a combination index, as described [36], and calculated as follows [37]:

$$\text{Combination index (CI)} = [(\%\ \text{NTS dead due to anthelmintic drug}) + (\%\ \text{NTS dead due to antioxidant})]/(\%\ \text{NTS dead in the presence of the drug combination}) \quad (1)$$

where the classification used has been described by other investigators [38], where CI > 0.1 very strong synergism, CI: 0.1–0.3 strong synergism, CI: 0.3–0.7 synergism, CI: 0.7–0.85 moderate synergism, CI: 0.85–0.9 slight synergism, CI: 0.9–1.1 nearly additive and CI > 1.1 antagonist. Each concentration of anthelmintic and anticancer drug, antioxidant either alone or combined was tested in duplicate, and the assays were performed at least twice.

3. Results

3.1. Anticancer Drugs Demonstrated Interesting Antischistosomal Activity in Comparison to Classic Anthelmintic Drugs

The NTS incubated with culture media only (without DMSO and compounds) exhibited normal viability with slight morphological alterations such as increased granularity and reduced motility. At completion of the incubation/assays, the NTS remained active with 92.8 ± 6.6% viable and score 2 according criteria of viability for phenotypic alterations (Table 1). The NTS incubated with DMSO (2%) presented similar viability, 92.4 ± 10.0% and morphological alterations. DMSO at 2% did not induce significant phenotypic alterations in NTS in comparison to NTS incubated with medium only. In Table 2 presents the mean and standard deviation (SD) percentage of NTS viability obtained during the different experiments on controls, vehicle control (DMSO), PZQ, and AS.

Table 2. Viability of NTS of *S. mansoni* (mean ± SD) at 72 h of exposure in vitro to praziquantel (PZQ), artesunate (AS), or DMSO (vehicle) at 100 µM.

% NTS Viability							
Control (w/o DMSO)	**Mean ± SD**	**Vehicle Control (DMSO)**	**Mean ± SD**	**PZQ**	**Mean ± SD**	**AS**	**Mean ± SD**
94.4	92.8 ± 6.6	69.4	92.4±10.0	34.8	50.0 ±23.9	0.0	87.5 ± 49.5
96.4		84.3		7.8		89.4	
95.3		96.4		52.0		87.5	
94.5		93.6		71.0		0.0	
100		100		50.0		93.8	
95.4		82.0					
78.8		78.2					
95.4		94.7					
94.7		92.7					

We evaluated two classes of drugs with discrete antischistosomal activity: the anthelmintics AS, PZQ and FBZ and the anticancer drugs IMT, and VDT at 100 µM. Viability of NTS following exposure to these compounds is presented in Table 3. Among the anthelmintics, FBZ was more active than AS and PZQ. Regarding to the anticancer agent's VDT was most potent against NTS leading to death of most NTS; only 29.4% remained alive (Table 3).

Table 3. Viability of NTS of *S. mansoni* (percentage) at 72 h of exposure to anthelmintic drugs (PZQ, AS, and flubendazole (FBZ)) and anticancer drugs (vandetanib (VDT) and imatinib (IMT)) at 100 μM in vitro.

Viability (%)					
Vehicle Control (Mean)	Anthelmintic		Anticancer		
	PZQ (Mean)	AS (Mean)	FBZ	VDT	IMT
92.4	50.0	87.5	14.8 0.0	29.4	77.3 72.3

PZQ at 100 μM induced several and severe morphological alterations following 72 h post-exposure. At one hour, the NTS were hyperactive with increased granularity and altered body shape. During the assay, the movement of NTS decreased and vacuoles and blebbing were induced (Figure 1). The NTS incubated with PZQ showed a mean phenotypic viability score of 1 or 0.5. Whereas the scoring system is semi-quantitative only, it does indicate that the NTS were severe altered although not dead. Indeed by 72 h some NTS remained alive and were actively moving. Moreover, the evaluation of viability using PI demonstrated that although they were severely altered, many NTS remained unstained and viable 50.0 ± 23.9%. The standard deviation was high due to inconsistent percentage of viability through assays with PZQ varying between 7.8 up to 71.0% (Table 1). It does not appear that drug lost activity since it continued to induce morphological alterations in NTS consistent with previous reports [35].

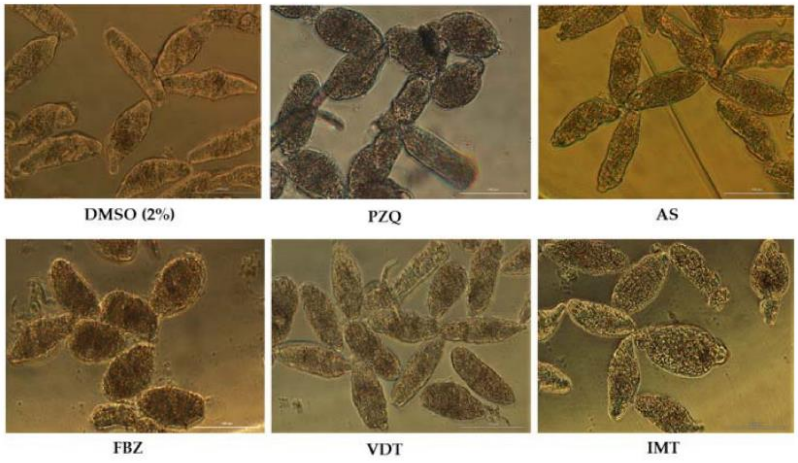

Figure 1. Representative micrographs of newly transformed schistosomula (NTS) at 72 h of exposure to the anthelmintics praziquantel (PZQ), artesunate (AS), and flubendazole (FBZ) and anticancer drugs vandetanib (VDT), and imatinib (IMT) at 100 μM. Compared to controls, NTS incubated with drugs showed severe morphological alterations, and the morphological changes differed among the drugs. Scale bar, 100 μm.

The NTS incubated with antimalarial and antischistosomal drug, AS at 100 μM showed inconsistent results (Table 2). In some assays, after 72 h the NTS presented dark body due severe granularity and no activity. Moreover, the tegumental membrane seemed to be disrupted. In these assays, all NTS were stained with PI and therefore were considered dead (0% viability). Curiously, in other assays performed under the same conditions, NTS did not show significant morphological alterations (Figure 1). Both the viability score or the viability assessed with PI were similar to controls. We hypothesized that stability of AS in solution decreased and consequently the drug might be degraded which could be translated in loss of antischistosomal activity, as reported by others [39]. Intriguingly, in a follow-up assay, the AS killed all the NTS following 72 h post-exposure; the mean and standard deviations reflected these uneven results (87.5 ± 49.5)%.

FBZ achieved better antischistosomal activity than PZQ and AS. Following 72 h post-exposure, NTS treated with FBZ had rounded up, were blebbed, exhibited minimal activity, severe granularity and altered (wrinkled) tegumental membrane (Figure 1). Viability as assessed with PI at 72 h revealed that mean of viability was 7.4%. The morphological alterations induced by FBZ were similar, although more marked, to PZQ. At least, both anthelmintic drugs induced the rounded phenotype. Although not all the NTS were killed by FBZ and by PZQ, these anthelmintics induced severe and irreversible morphological alterations. Therefore, we could speculate that the development from schistosomulum to adult parasite would be compromised.

Concerning the anticancer agents, VDT and IMT showed potent to moderate activity against NTS at 100 μM. During evaluation of VDT at 100 μM, the morphological alterations were more pronounced at 72 h exposure, displayed altered shape, increased granularity and lack of movement at lower magnification (Figure 1). Assay of viability with PI revealed that VDT induced death in most (~70%) of the NTS, while those remaining alive showed minimal or no movement. VDT at 25 μM showed reduced antischistosomal activity; at 72 h exposure, VDT induces slight morphological alterations similar to those observed to controls, and percentage viability assessed with PI was not different from the controls (not shown). IMT showed moderate activity. By 72 h, morphological alterations in NTS included granularity (Figure 1). The majority of NTS remain alive and unstained by PI (72.3% viable) and viability score of 2 (Tables 1 and 3). NTS incubated with IMT was more active and less altered than VDT.

3.2. Antioxidants Alone Showed Potent Antischistosomal Activity

Eight antioxidants were studied: Resv, Flav, Curc, Mel, Kaempf, OXA, H-L-Trp-L-Ser-OH (DiPept), and NAC. All were evaluated at 100 μM alone and combined (see below) with the drugs listed earlier. Findings for antioxidants alone shown in Table 4.

Table 4. NTS viability (%) of NTS after exposure for 72 h to antioxidants alone at 100 μM, as assessed by staining with PI.

Viability (%)								
Control	**NAC**	**Resv**	**Flav**	**Curc**	**Mel**	**OXA**	**Kaempf**	**DiPept**
92.4 (mean)	92.2	43.1 64.7	59.3 89.4 67.6	0.0	93.1 98.1	0.0	ND [1]	88.9 93.8

[1] ND: not done.

Two antioxidants, Curc and OXA, demonstrated a striking antischistosomal activity, killing all NTS before the end of experiment. The morphological alterations induced by Curc and OXA were dissimilar, suggesting different modes of action. At one-hour exposure to Curc, the schistosomula showed severe morphological changes including severe granularity, altered shape. Curc killed all NTS by 1 h. NTS incubated with OXA at the same period did not present significant morphological alterations, but they had no movement. Following 17 h exposure, NTS incubated with OXA showed increases granularity, blebbing, and lack of movement. Figure 2 shows NTS incubated with the eight antioxidants at 72 h. At the end of experiment, viability of NTS was assessed and confirmed by staining with PI.

Among the other antioxidants, Resv and Flav exhibited moderate antischistosomal activity, and significant morphological alterations (Figure 2). Following 72 h post-exposure, Flav had caused severe granularity and minimal activity in NTS, corresponding to viability score of 1 (Table 1). Despite the morphological alterations, 59.3% NTS remained alive. Resv also induced morphological alterations into NTS, although not as pronounced as by Flav. Most NTS incubated with Resv had severe granularity, minimal activity, alteration of shape. Some exhibited only mild granularity and no alteration of shape. These NTS had a score viability of 1.5 and 43.1% viability when assessed with PI (Table 4).

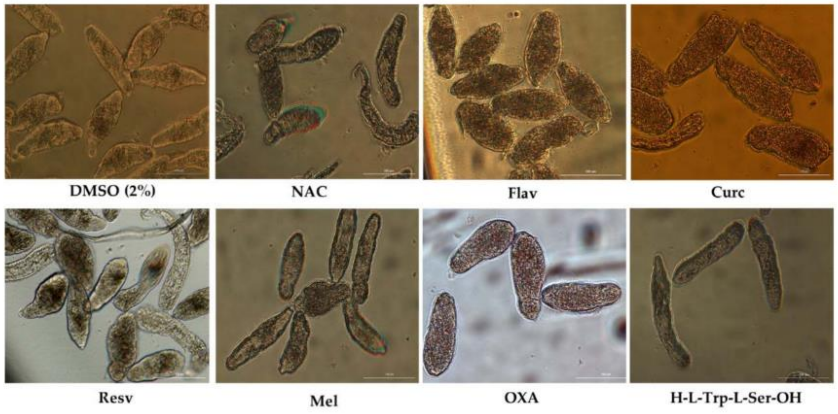

Figure 2. Representative micrographs of NTS at 72 h of incubation in antioxidants at 100 μM. Notable differences were evident among controls and NTS incubated with 4-phenyl-1,2,5-oxadiazole-3-carbonile-2-oxide (OXA), curcumin (Curc), and flavone (Flav), which induced severe granularity and reduced activity. In contrast to OXA and Curc, Flav did not kill all the NTS. Resveratrol (Resv) also induced moderate morphological alterations but did not kill all NTS. *N*-acetylcysteina (NAC), melatonin (Mel) and dipeptide (DiPept) did not induce significant morphological alterations. Scale bar, 100 μm.

The antioxidants, Mel, DiPept and NAC failed to cause significant antischistosomal effects. At 72 h, NTS had a percentage of viability similar to controls (~90%) (Table 4). The NTS remained viable during the experiment without significant morphological alterations and only marginal increase of granularity and decrease of activity, with a viability score of 2.5. Notably, and by contrast, following 72 h exposure to DiPept, NTS were more active than controls suggesting that this antioxidant might enhance maintenance of NTS in vitro. Evaluating the antischistosomal activity of Kaemp was not feasible because a precipitate formed upon addition of the stock to wells containing NTS in culture medium, evidently the result of interaction of Kaempf with proteins in culture medium [40]. The NTS exhibited morphological alterations, but we could not ascribed the effects to Kaempf due to the obvious perturbation of the culture medium.

3.3. Antischistosomal Activity of Combination of Drugs and Antioxidants Show That Antioxidants Might Enhance Activity of Drugs

Among the combinations of PZQ with antioxidants, PZQ + Curc and PZQ + OXA achieved better results leading to death of all NTS before 72 h of exposure in a similar fashion to observed with these antioxidants alone (Table 5). Therefore, the potent antischistosomal activity observed in combinations was related to the antischistosomal activity of antioxidants itself. The morphological alterations observed with these combinations were more similar to those induced by antioxidants alone rather PZQ (Figure 3). Intriguingly, the combination index demonstrated that PZQ combined with Curc or OXA exhibited slightly antagonism (Table 5).

Table 5. Viability of NTS (%) following exposure to PZQ, antioxidant and combination and its cooperative index.

	Viability (%)			Combination Index
	Anthelmintic	AntiOx	Combination	
PZQ+Resv	34.8	43.1	3.5	0.74
PZQ+Flav	7.8	59.3	72.9	4.90
PZQ+Curc	52.8	0.0	0.0	1.47
PZQ+Mel	52.8	93.1	31.4	0.80
PZQ+Kaempf	71.0	ND	ND	ND
PZQ+OXA	71.0	0.0	0.0	1.29
PZQ+DiPept	50.0	93.8	58.9	1.37
PZQ+NAC	50.0	92.2	59.3	1.42

ND: not done.

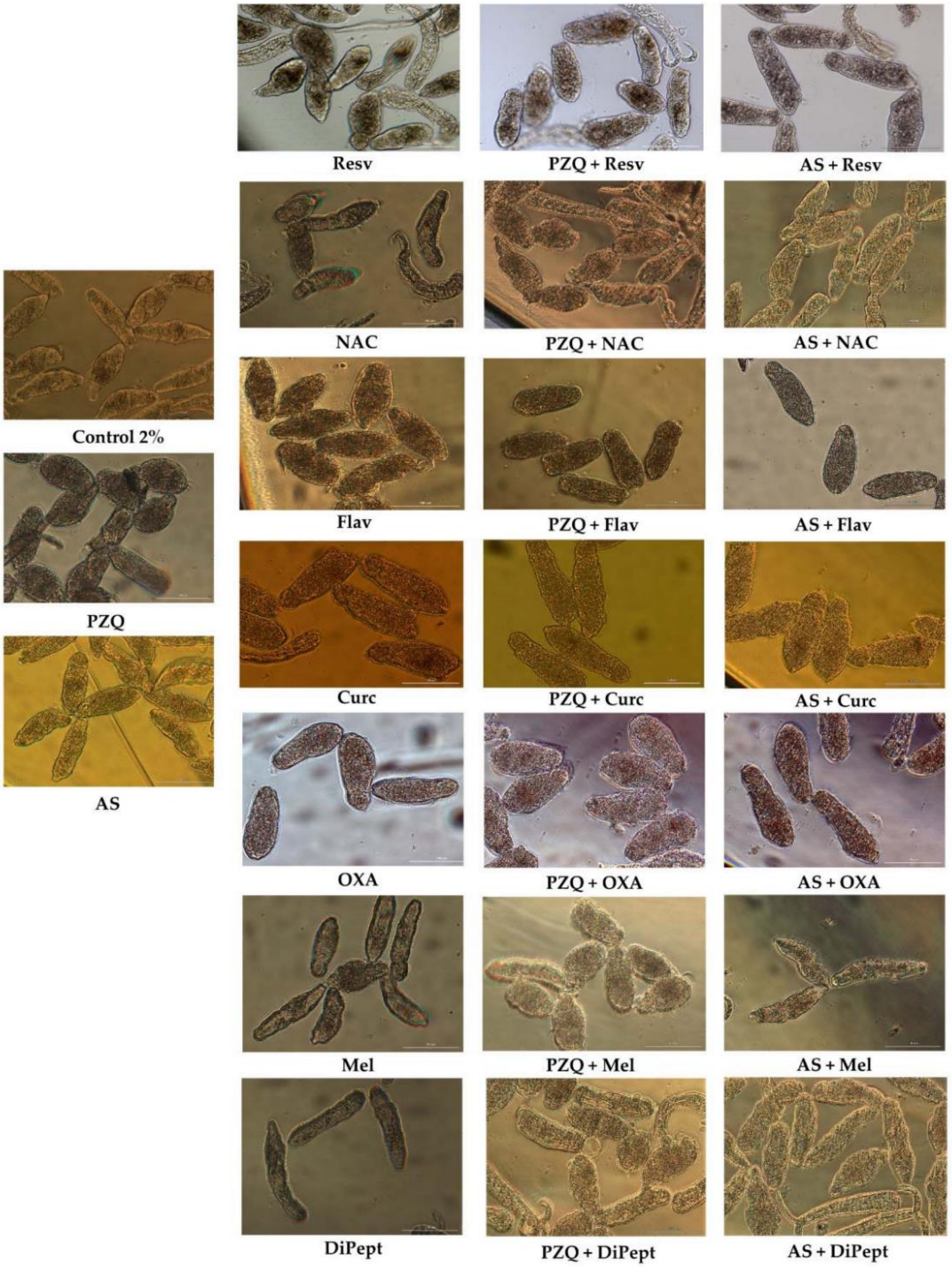

Figure 3. Representative micrographs of NTS at 72 h after exposure to combinations of praziquantel (PZQ) and artesunate (AS) with several antioxidants at 100 μM, and 1:1 constant ratio; scale bar, 100 μm.

The antioxidants Mel and Resv enhanced antischistosomal activity of PZQ. The combination of these antioxidants, i.e., PZQ + Mel and PZQ + Resv leads to a significant decrease of NTS viability in comparison to either compound alone. In fact, the combination index demonstrated that they are slightly synergistic. The larvae incubated with combinations had similar morphological alterations induced by PZQ alone (Figure 3); nonetheless, they were less active and viability was lower than for the compounds alone (Table 5).

The NTS treated with combinations of PZQ with Flav, DiPept, and NAC achieved better percentage of viability than compounds alone. Comparing the viability of PZQ (7.8%) and Flav (59.3%) alone with PZQ + Flav it was possible to observe a significant increase (72.9%). Apparently, Flav suppresses the antischistosomal activity of PZQ acting as antagonist as expresses by CI (Table 5). Despite the percentage viability in PZQ + Flav, NTS were severely damaged and minimum activity was detected (Figure 3), yet, they remained alive—they did not stain with PI (not shown). This suggest that compounds did not damage the tegumental membrane of the NTS. Comparing morphological alterations of NTS incubated with compounds alone or combined, some differences were apparent among them. The morphology of NTS incubated with PZQ + Flav was different from those incubated with compounds alone (Figure 3). The body shape was marginally different from Flav being more similar to PZQ. All NTS had severe granularity and minimal activity, in accord with the viability score of 1 (Table 1).

The antioxidants DiPept or NAC combined with PZQ slightly improved viability of NTS in comparison to drug alone. Taken into consideration the CI, we classified these combinations as additive (Table 5). The morphological alterations observed in NTS treated with these combinations were similar to those observed with PZQ (Figure 3), but the viability score was 1 rather 0.5 attributed to PZQ alone. Also, the percentage of viability assessed with PI revealed that in combinations were slightly higher than PZQ alone (Table 5). As noted above, this may relate to the fact that these antioxidants might enhance viability of NTS in vitro.

In general, combinations of AS with antioxidants were more active than the compounds alone. In like fashion to findings described above, exposure for one or 17 h to combinations AS + Curc and AS + OXA caused the death of NTS, respectively. The effects were related with activity of antioxidants alone rather than to enhancement of antischistosomal activity of AS. These two combinations were classified as nearly additive (Table 6). The morphological alterations observed in NTS were similar to those described for antioxidants alone (Figure 3), supporting the notion that antischistosomal activity was due to antioxidants alone and not to AS.

Table 6. Viability (%) and cooperative index for NTS exposed to AS, antioxidant and the combination.

	Viability (%)			Combination Index
	Anthelminthic	AntiOx	Combination	
AS+Resv	0.0	43.1	0.0	0.34
AS+Flav	89.4	59.3	49.2	1.01
AS+Curc	87.5	0.0	0.0	1.12
AS+Mel	87.5	93.1	95.7	4.51
AS+Kaempf	0.0	ND	ND	ND
AS+OXA	87.5	0.0	0.0	1.12
AS+DiPept	93.8	93.8	88.0	1.03
AS+NAC	93.8	92.2	84.3	0.89

ND: not done.

The AS + Resv combination resulted in death of all NTS by 72 h following drug exposure whereas AS alone was not lethal to all the NTS. Comparing the morphological alterations induced by AS and Resv alone and combined, e.g. AS + Resv, it was clear that they were more pronounced in NTS treated with the combination. It is noteworthy that the membrane of NTS was compromised and disrupted (Figure 3). Assessing viability by PI staining revealed that all NTS treated with the combination were stained (0% viability) in contrast to incubation with AS and Resv alone (Table 6). Accordingly, Resv enhanced antischistosomal activity of AS, as previously observed [30].

In contrast to PZQ + Flav, the combination of AS + Flav reduced viability almost to half of viability observed in AS alone. This reduction was less pronounced when comparing viability of Flav and AS + Flav (Table 6). It is interesting to note that the same antioxidant behaved in a different manner when combined with different drugs. The morphological alterations observed in NTS treated with AS + Flav were similar to those induced by Flav alone; however, NTS shape was more oval (Figure 3). Nonetheless, both NTS incubated with Flav and AS + Flav had minimum, almost undetectable activity

and their viability score was 0.5. By contrast, with AS, movement was apparent as were slight morphological alterations consistent to viability score of 2. In fact, the viability assessed by PI confirms these observations, as we can observe on Table 6, Flav had a lower viability compared to AS alone. Similarly, in combination, the viability was also lower than compounds alone. This combination was classified as nearly additive (Table 6).

The combination of NAC or DiPept with AS resulted in decreased viability compared to the compounds alone; however, the reduction was not pronounced (~10%) and morphology of NTS were similar to controls. In general, NTS did not present significant alterations besides slight increase of granularity and decrease of activity. Regarding AS + DiPept, NTS were less active than those incubated with DiPept alone. This combination was classified as additive while AS + NAC was slightly synergistic (Table 6). Interestingly, NTS incubated alone or in combinations presented same phenotypic viability score of 2.

The combination of AS + Mel was considered as antagonist, in contrast to PZQ + Mel. In this case, NTS incubated with AS + Mel presented slightly better viability compared to the compounds alone (Table 6). Similar to combinations with NAC or DiPept, NTS incubated with AS + Mel did not present any significant morphological alterations, only a slight increase of granularity and decrease of activity but remained viable with a similar phenotypic viability score (2.5) and percentage (~90%) to those of the controls and compounds alone (Figure 3; Table 6).

FBZ was evaluated in combination with the antioxidants, DiPept, Resv, and Flav. These antioxidants were selected based on the results obtained in the combinations with PZQ and AS. Table 7 presents the viability (%) of NTS after exposure to combinations with FBZ and the combination index.

Table 7. Viability (%) of NTS following exposure to FBZ, antioxidant alone and the combination, and the cooperative index for each combination.

	Viability (%)			
	Anthelmintic	**AntiOX**	**Combination**	**Combination Index**
FBZ + Resv	0.0	64.7	0.0	1.35
FBZ + Flav	0.0	67.3	0.0	1.32
FBZ + DiPetp	14.8	93.8	95.7	21.25

Notably, combining FBZ and DiPept improved survival of the NTS compared to FBZ alone (Table 7). This is intriguing, but similar to observations for the combination PZQ + DiPept, although less pronounced. However, NTS incubated with FBZ + DiPept following exposure for 72 h did not show detectable activity. Nonetheless, most were still alive since they did not stain with PI (viability, 95.7%). The morphological alterations induced by combination were similar to NTS incubated with FBZ alone: a rounded shape with tegumental blebbing (Figure 4). The outcome was similar to that for PZQ and Flav alone; severe alterations but many remained viable. Also, DiPept might be important for the maintenance of the tegument and its membrane and to prevent the entry of PI. According to the CI, this combination was highly antagonistic (Table 7).

When FBZ was combined with Resv or Flav, NTS were dead by 48 h after exposure in both cases, as confirmed by PI staining. This outcome likely was due solely to drug since all NTS incubated with FBZ alone also died (Table 7). Nonetheless, the morphological alterations induced by combinations FBZ + Resv and FBZ + Flav were different. The NTS incubated with FBZ + Flav displayed an oval form and darker body, similar to FBZ alone.

On the other hand, NTS expose to FBZ + Resv were more elongated with severe granularity which is more consistent with morphological alterations induced by Resv (Figure 4). Probably, Resv reverses the morphological alterations induced by FBZ but did not affect its activity given that all the NTS subsequently died. Curiously, both combinations, FBZ + Resv and FBZ + Flav, were classified as marginally antagonistic (Table 7).

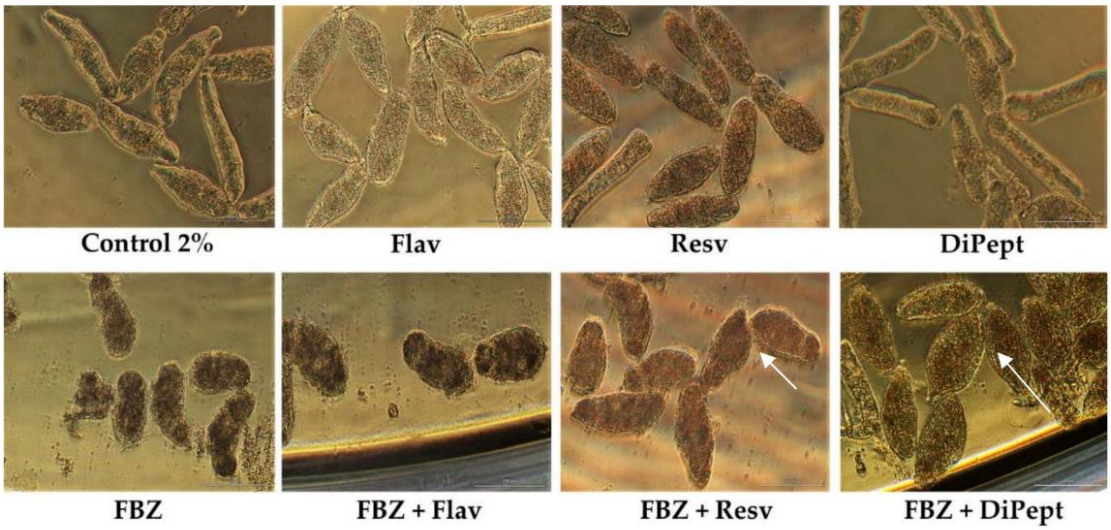

Figure 4. Representatives micrographs of NTS incubated with combinations of FBZ with Flav, Resv, and DiPept at 100 μM and constant ratio 1:1 at 72 h post-exposure. Notably, the morphologic alterations in combinations were more similar to drug alone rather antioxidant, specifically FBZ + Flav. In combinations FBZ + Resv and FBZ + DiPept NTS were more swollen and altered in shape (white arrows). Scale bar, 100 μm.

We also evaluated antischistosomal activity against NTS of anticancer drugs, VDT, and IMT combined with several antioxidants. The results achieved by anticancer drugs in combination with antioxidants are listed in Table 8.

Table 8. Viability of NTS (%) following exposure to anticancer drugs, antioxidants, drug combinations, and combination indexes.

	Viability (%)			
	Anticancer	**AntiOx**	**Combination**	**Combination Index**
VDT + Flav	29.4	67.6	0.02	1.03
VDT + Resv	29.4	64.7	0.09	1.06
IMT + Flav	77.3	89.4	77.1	1.45
IMT + Resv	72.3	64.7	64.1	1.75
IMT + Mel	77.3	98.1	91.3	2.83

The anticancer drug, VDT was evaluated combined with Flav and Resv. As we mention above the drug itself and antioxidants (Flav and Resv) had antischistosomal activity against NTS. Interestingly, the combination of VDT with Resv and Flav resulted in a significant increase of antischistosomal activity leading to death of NTS. The percentage of viability achieved by these combinations was near 0% (Table 8). In fact, it is clear that the combination of these antioxidants with VDT reveal additive effect as confirm by CI depict on Table 3. The morphology of NTS incubated with combinations was different than on compounds alone and control (Figure 5). In case of VDT + Flav, NTS were neither similar to Flav alone or VDT while in VDT + Resv they were more similar to Resv alone although more oval shape rather vemiform.

We also evaluated the combination of VDT with Curc or OXA at constant ratio of 1:1 but at a lower concentration, 25 μM. In both combinations, VDT + Curc and VDT + OXA, all the NTS were dead at the end of experiment due to antischistosomal activity of the antioxidants (see above) and not due to anticancer drug. The combinations of VDT + Curc and VDT + OXA killed all NTS following 1 h exposure to the antioxidants alone. This is related to antischistosomal activity of anioxidants that alone also killed all the NTS at the same time (above). In these combinations, NTS showed similar morphological alterations to those induced by antioxidant alone (Figure 6).

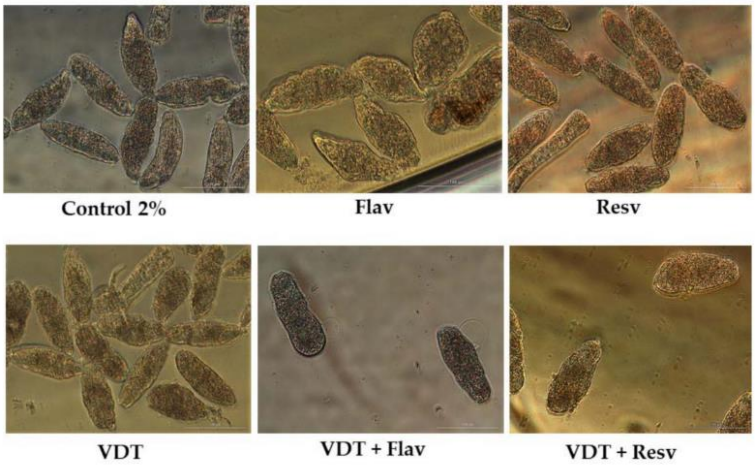

Figure 5. Representative micrographs of NTS at 72 h of incubation in VDT, Flav and Resv either alone or combined at 100 μM and 1:1 constant ratio; scale bar, 100 μm.

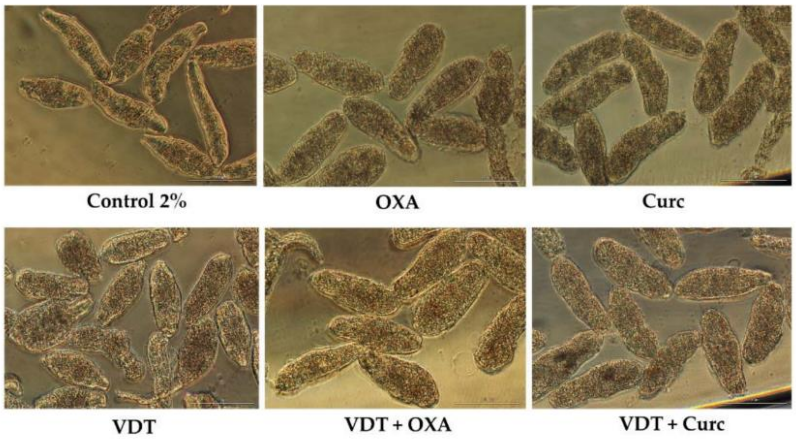

Figure 6. Representative micrographs of NTS incubated with combinations of VDT, Flav, and Resv alone and in combination at 25 μM and 1:1 constant ratio at 72 h post exposure. Scale bar: 100 μm.

With IMT, we evaluated its combination at constant ratio 1:1 and 100 μM with Flav, Resv, and Mel. Following 72 h exposure, IMT combined with Flav and Resv did not enhance antischistosomal activity. NTS treated with combinations had similar viability to anticancer drugs or antioxidants alone (Table 8). Nonetheless, NTS treated with combinations induced morphological alterations that in the case of IMT + Flav were consistent with Flav alone although with more granulation. With IMT + Resv, NTS had severe granularity and altered, swollen body shape (Figure 7). The combination indexes for IMT + Flav and IMT + Resv suggested that they are slightly antagonist.

In contrast, the combination of IMT + Mel resulted in increase of percentage of viability (91.3%) in comparison to IMT alone (77.3%) and a slight decrease when compared to Mel alone (98.1%) (Table 8). Nonetheless, NTS showed morphological alterations consistent to those induced by IMT alone. Some of NTS were round and swollen (white arrow) while others remained elongated but different from Mel alone, whereas Mel did not revert the morphological alterations, it rescued NTS from death.

The Combination of Two Antioxidants (OXA + Curc) is Highly Potent Against NTS

In order to understand whether Curc or OXA was the more potent antioxidant, we conducted an in vitro assay to evaluate the combinations against NTS at the lower concentration of 25 μM. However, even at this lower concentration we could not determine which one was more potent since both killed all the NTS by 17 h after exposure. Curcumin induced more morphological alterations and impaired movement earlier than OXA. At one hour, similar to 100 μM, NTS incubated with OXA was more

similar to controls whereas NTS in Curc had already altered. In combination, all NTS were dead by 17 h following exposure. The morphological alterations of NTS in Curc + OXA were more similar to those induced by OXA than Curc (Figure 8).

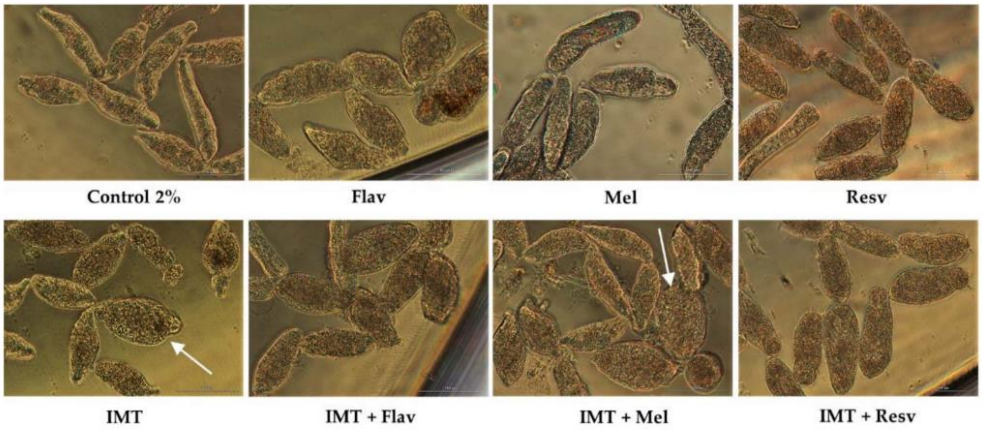

Figure 7. Micrographs of NTS at 72 h of exposure to IMT, Flav, and Resv, Mel alone or combined at a concentration of 100 μM and 1:1 constant ratio; x20; bright field; scale bar, 100 μm.

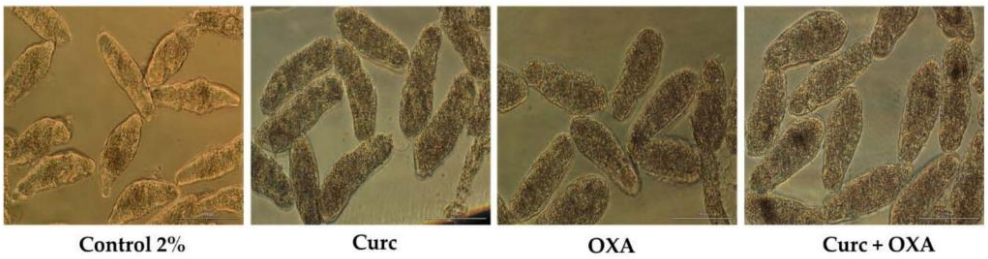

Figure 8. Representative micrographs of NTS at 72 h of exposure to Curc and OXA and combined, at 25 μM and 1:1 constant ratio; ×20; bright field; scale bar, 100 μm.

Nonetheless, the cooperative index for this combination was two, which indicated that these drugs were antagonists. This was unexpected and anomalous to what we had observed. In fact, all NTS were killed either by antioxidants alone but also by combination in a similar time of exposure. Note that dead of all NTS either incubated with antioxidants alone or in combination was confirmed through staining of PI.

4. Discussion

The current treatment for schistosomiasis based only on PZQ [2] cannot fully counteract the infection associated morbidity and sequelae including periportal fibrosis, esophageal varices, and, with *S. haematobium* infection, sandy patches of the lower female genital tract [41], and squamous cell carcinoma of the bladder [5,12,42]. In the present study, we propose a novel therapeutic strategy that combined principles of drug repurposing and the combination of different active agents. Drug repurposing is based on previous investigations and given that some drugs are already commercialized, novel antischistosomal drugs could quickly advance into clinical testing, diminishing the time and cost of new drug development [7]. Our aim is achieved synergistic and/or additive effect that not only eliminate the parasite but also ameliorate morbidity associated to infection. Here we evaluated the ability of biomolecule antioxidants to enhance antischistosomal activity of anthelmintic and anticancer drugs against the schistosomulum stage of *S. mansoni* in vitro. These biomolecules were investigated due to their biological properties and the possibility of antischistosomal activity per se [16].

We evaluate three anthelmintic, PZQ, AS, and FBZ known to be active against different developmental stages of the *Schistosoma* species and other parasites. Whereas PZQ is more effective

against adult worms, AS is more active against larval stages [43]. The anthelmintic drug FBZ has broad spectrum action against tapeworm infections. In vivo studies (in mice) demonstrated its activity in reducing numbers of adult parasites of *S. mansoni* [44,45]. Here, FBZ was more potent than PZQ or AS against NTS. Therefore, FBZ not only is active against adult worms but also against the schistosomulum stage. Based on in vitro and in vivo data it seems that FBZ is active against several developmental stages of *S. mansoni*. This antischistosomal activity is of interest since it suggests that it might overcome one of the limitations of PZQ and even AS. The mechanism of action of FBZ encompasses its ability to specifically bind and interact with microtubules that are main components of the eukaryotic cytoskeleton [46]. Probably FBZ interacts with microtubule inhibiting the surface membrane maturation of NTS and consequently altered its shape and leading to death [47]. In regard to the mechanism of action of AS and PZQ, they remain uncertain, but AS action may be related to the presence of endoperoxide bridge that induces the production of ROS whereas PZQ might act through the calcium channels [2,43].

The three anthelmintic drugs induced different morphological alterations which is probably related to its different targets on NTS and to their discrete modes of action. Nonetheless, these morphological alterations are more similar between PZQ and FBZ compared to AS. Both drugs induce an oval shape worms (rather than vermiform) and severe granularity. However, reduction of viability was more pronounced with FBZ. Although PZQ and FBZ induces severe morphological alterations on NTS, they did not kill the NTS, consistent with other reports [48].

We also evaluated the anticancer drugs IMT and VDT which are kinase inhibitors. The importance of kinases in members of the family Schistosomidae has been reviewed extensively [49]. Schistosome kinases play pivotal roles for different physiological processes, including reproduction, which is closely associated with egg production and the pathology of schistosomiasis. The anticancer drugs alone had demonstrated moderate to potent activity against NTS. Most likely the antischistosomal activity of IMT and mostly VDT are linked to the fact that they might impair the kinases of NTS which are very important to their development [49]. In next phase, we will assess which schistosome kinases are possible affected by these anticancer drugs.

The use of antioxidants against schistosomiasis have been reviewed [16]. Here we selected several antioxidants and evaluated not only antischistosomal activity but also its ability to enhance antischistosomal activity of both anthelmintics and anticancer agents. Of the antioxidants evaluated, Curc and OXA were highly active, and quickly killed the schistosomula. These findings were consistent with others reports [50]. Curcumin has demonstrated antischistosomal activity in vitro against adult worms and also affects the development of eggs [51]. The mechanism by which Curc exerts in vitro antischistosomal effect against both larval and adult worms is uncertain. However, Curc has a direct action involving in parasite biochemical processes. One possible target is the ubiquitin-proteasome pathway [52]. Proteasome inhibitors reduce the number of lung stage schistosomula, the worm burden and consequently decrease the egg output in infected mice [50,51]. Therefore, it is reasonable to hypothesize that in vitro antischistosomal activity of Curc against NTS might be due to inhibition of the ubiquitin-proteasome pathway. Regarding OXA, antischistosomal activity against NTS might be related to its ability to inhibit thioredoxin glutathione reductase (TGR) [19]. This enzyme is essential for parasite survival and is biochemically distinct from host enzymes; also, the parasite redox system is dependent of TGR [53]. Inhibition of TGR activity would lead directly to the inactivation of both thioredoxin and glutathione-based defenses and the accumulation of ROS and RNS species [19]. The results obtained for these two antioxidants suggested that antischistosomal activity might be related to different modes of action. The combination of these two antioxidants at lower concentrations also translated into potent antischistosomal activity. The morphological alterations induced by combination of antioxidants were different in comparison to those induced by antioxidant alone.

Flav and Resv demonstrated similar moderate antischistosomal activity against NTS. The results obtained for Resv were consistent to findings that we reported previously [30]. Comparing the morphological changes induced by the two antioxidants, they were more pronounced in NTS incubated with Flav. Resv might act on neuromotor activity based on its effects on motility, which in turn could

degrade its ability to migrate and acquire nutrients [30]. It is worth emphasizing that in experimental schistosomiasis in murine model Resv ameliorates oxidative stress and organ dysfunctions [54]. Since Resv had moderate antischistosomal activity here, maybe the results obtained by Soliman et al. are not related, not only due to its biological properties but also its antischistosomal activity against parasites. The mechanism of action of Flav against NTS is unclear. Flav has shown a broad spectrum as an anticancer and antioxidant, among other attributes. Its anticancer effects are exerted through binding receptor estrogens [25]. This is interesting since *Schistosoma* spp. produce/excrete estrogen-like metabolites to host that triggers a cascade of events that culminate in cancer in the case of *S. haematobium* infection [12,13]. Maybe the effects of Flav in NTS are associated to these metabolites or its inhibition. It is unclear why and how these metabolites are acquired and their role in parasite. Some authors attribute broad spectrum activities of Flav to their capacity to modulate key cellular enzyme function [55]. Further studies are necessary to evaluate its antischistosomal activity against adult worms and the possible drug targets.

In contrast, Mel, DiPept and NAC did not exhibit significant antischistosomal activity. Interestingly, NTS incubated with these antioxidants were more active than controls. These compounds likely are necessary for NTS maintenance, at least in vitro. Other reports demonstrated Mel also did not present antischistosomal activity against adult worms [56]. Nevertheless, some studies reported that they might ameliorate morbidity associated to infection. For example, NAC and Mel in vivo studies ameliorate redox homeostasis by downregulating oxidative stress caused by infection reducing fibrotic area and granulomas [56,57]. Mel might be use in immunization program, also might has multiple direct and indirect antioxidant actions as ability to stimulate host antioxidant enzymes and mitochondrial oxidative phosphorylation [58]. This antioxidant might be responsible for the immunoprophylactic effect and could protect host against infection [16]. In the case of DiPept, its antischistosomal activity was evaluated for the first time. Despite, it did not demonstrate antischistosomal activity it might be useful in ameliorate of pathology. NTS incubated with DiPept were more active rather controls, an outcome that might be related to the fact that dipeptides are building blocks of proteins and thus, the acquisition of these small peptides is critical for protein anabolism necessary for larvae in vitro. Regarding Kaempf it was impossible evaluate its antischistosomal activity since this antioxidant interacts with proteins presented in culture media precipitating [39]. For evaluation of its activity it would be necessary to use fetal bovine serum free medium which might compromise NTS viability.

The results obtained by combinations were interesting and demonstrated that even though some antioxidants did not display significant activity against NTS, that does not necessarily indicate that they were not able to enhance antischistosomal activity of drugs. Interestingly, this effect was more pronounced in the case of combinations with AS and anticancer VDT. In terms of combination index, combinations of AS or VDT with antioxidants achieved better results with most of them classified as additive or synergistic. Based on these results, it seems that AS and VDT present a better profile for the combination of different active agents preventing the nullification of the activity of one compound over the other.

Nonetheless, PZQ combined with Resv or Mel were also classified as synergistic. The synergism in antischistosomal activity could be a result from increased action against anthelmintic drug targets or by acting concomitantly on discrete targets [59]. By contrast, some combinations were classified as antagonists including PZQ + Flav, PZQ + OXA, PZQ + DiPept, FBZ + Resv, FBZ + Flav, IMT plus Flav or Resv and AS + Mel. Although they were classified as antagonistic it should be noted that it a slightly antagonist (CI between 1.75 and 1.29). In most of these cases, the percentage of viability for NTS incubated with combinations slightly increased in comparison to the compounds alone. Yet, it does not necessarily mean that combinations of drugs plus antioxidant annuls the effect of each other. In the case of combinations such as PZQ + Flav, AS + Mel, FBZ + DiPept, and IMT + Mel which exhibited CI values ranging 2–21, the percentage of viability in NTS incubated with combinations increased comparatively with compounds alone. We hypothesize that combinations of the compounds might inhibit antischistosomal activity of either the drug or the antioxidant. The same antioxidant incubated with different drugs displayed different combination indexes. The antimalarial AS combined with Mel

presented a CI = 0.80 suggesting synergism. Interestingly, a combination of the same antioxidant with a different drug, such as IMT or Mel, did not enhance antischistosomal activity of drug. Indeed, the CI revealed that they acted as antagonists. In a similar fashion, DiPept combined with AS achieved an additive effect; however, in combination with FBZ and PZQ, DiPept acts as an antagonist (more pronounced in FBZ + DiPept). In this case, it seems that the antioxidant inhibits the antischistosomal activity of drug. As mentioned above maybe NTS requires DiPept for their maintenance in laboratory culture or that interaction of both compounds inhibits FBZ activity. These findings suggested that the activity of the antioxidant varies depending on the drug used for the combination. In general, morphological alterations were more pronounced in NTS treated with combinations rather compounds alone, even if it did not result in their death. Most likely, severely altered NTS could not growth and develop into adult worms. Accordingly, this strategy might be of value for elimination of schistosomes, at least, the larval stage of *S. mansoni*. Further studies, including in vivo studies using the rodent model of schistosomiasis are required for evaluation of its efficacy not only against adult worms but also in amelioration of morbidities associated with the infection.

5. Conclusions

Not only did the antioxidants exhibit antischistosomal activity but several antioxidants enhanced the schistosomical activity of the anthelmintics drugs. The additive or synergistic effects achieved by combinations of antioxidant and anthelmintic might be related their different mode of action and/or different targets on NTS. Repurposing of drugs as FBZ or anticancer drugs (or indeed other classes) might be worthwhile since they were effective against the schistosomulum stage of *S. mansoni*. This approach might be considered for prophylaxis or for use in regions with intense re-infection levels since the combination might block or retard parasite infection and development. Investigation of effects can now proceed to confirm the synergies of these combinations reported here against adult forms, and in vivo in laboratory rodents and likewise in other schistosome species including *S. haematobium*. Due to the biological properties of antioxidants in prevention of DNA damage and blocking cancer initiation processes [21–25], antioxidants may also counteract carcinogenesis during infection with helminth parasites [12,13,60,61].

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Chapter 6

Combination of antioxidants with drug repurposed against adult worms of Schistosoma mansoni

CHAPTER 6. Combination of antioxidants with drug repurposed against adult worms of *Schistosoma mansoni*

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The purpose of research work presented in this Chapter was evaluated the novel therapeutic approach against adult worms of *S. mansoni.* During this research work we investigate the antischistosomal activity of anthelmintic, anticancer drugs and antioxidants alone and combined. For that purpose, the adult worms were recovered from experimentally infected mice by perfusion of portal veins and used for *in vitro* antischistosomal activity assay as described during this Chapter. Additionally, the effect of drugs and antioxidants either alone or combine on oviposition was also evaluated. Similarly, to observed for NTS, antioxidant biomolecules by itself presented an interesting antischistosomal activity and also enhance antischistosomal activity against adult worms. Interestingly, anticancer drugs also induce high mortality of adult worms *in vitro*. Most of the drugs evaluated as some antioxidants cease the oviposition of adult worms either by its death or possible damage in reproduction apparatus of female. Taken together these results, the concepts of drug repurposing and combination of agent with different mode of action might be relevant to prevent sequelae associated to infection since eggs are one of the major factors for inflammatory processes associated to helminth infection.

Combination of antioxidants with drug repurposed against adult worms of *Schistosoma mansoni*

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Abstract

Despite the efforts to control and eliminate schistosomiasis, this neglected tropical disease remains a major public health problem. Currently, the treatment for schistosomiasis relies on a single drug, praziquantel (PZQ). Although PZQ is effective against the adult worms, its present major drawbacks including inefficacy against the juvenile and alone is not capable to counteract infection-associated disease lesions including hepatic fibrosis. Moreover, there is a legitimate concern about the emergence of PZQ-resistance. Therefore, there is a pressing need for novel therapies that not only target parasite but prevent or decrease infection-associated pathologies. Antioxidants present interesting biological properties that might render them a valuable tool for schistosomiasis. Previously, we observed synergistic/additive effect through the combination of drugs and antioxidants against newly transformed schistosomula (NTS) of *Schistosoma mansoni*. Here, we investigated this novel therapy approach against adult worms of *S. mansoni*. The findings indicate that some antioxidants showed antischistosomal activity per se against adult worms, and slightly improve the antischistosomal activity of drugs when combined. Additionally, the novel therapeutic approach prevents oviposition which could be crucial to counteract the pathologies associated with infection. Eggs released from parasites are directly linked to the formation of granuloma and inflammation associated with parasitic infection. Therefore, these evidences could be important since the manipulation of the rate of oviposition might eventually lead to new approaches for disease control.

Keywords: *Schistosoma mansoni, adult worms, antioxidant biomolecules, drug repurposing, combination therapy, oviposition.*

1. Introduction

Schistosomiasis is one of the most important neglected tropical diseases. The infectious agents are helminth parasites of genus *Schistosoma* (WHO, 2012), and three main *Schistosoma* species including *Schistosoma mansoni*, *S. haematobium* and *S. japonicum*, are responsible for human schistosomiasis. Only *S. haematobium* is considered carcinogenic and responsible for about 90% of infections in Africa Sub-Sahara and (WHO, 2012). Schistosomiasis is one of the most widespread parasitic infection with high levels of incidence in Asia, Africa and Latin America affecting 207 million people and about 779 million are at risk of infection (WHO, 2012). Recently, outbreaks of schistosomiasis haematobia have been reported in Western Europe (Berry et al., 2014; Boissier et al., 2016).

For over more than 40 years, the control and treatment of schistosomiasis rely on a single drug, praziquantel (PZQ) (Vale et al., 2017). Despite its efficacy against all forms of human schistosomiasis and low toxicity, the drug has major drawbacks as a limited effect on juvenile schistosomes and on liver a spleen lesion as a consequence of infection and liberation of eggs by adult worms (Sabah et al., 1986). Moreover, due to the extensive and long-term repeated use of PZQ, there is a growing and legitimate concern about the development of drug resistance or reduced susceptibility (Vale et al., 2017). Thus, there is a consensus on the urgent need to develop novel, affordable and effective therapies against this debilitating parasitic disease. Drug repurposing and/or combination of distinct biological active agent might be an efficient approach to reduce time and cost of drug research and development (Keiser et al., 2011; Cowan and Keiser, 2015). The current therapy for schistosomiasis mainly targets the parasite and not the pathologies associated with the infection (Vale et al., 2017). During the host immune response to the presence of the parasite occur the liberation of reactive oxygen species (ROS) which might disturb the cellular antioxidant homeostasis of affected organs (Maizels et al., 1993; Gharib et al., 1999). Regard to S. haematobium infection our research group suggested that reactive electrophilic compounds, e. g. estrogen-like metabolites might act as potential initiators of infection-associated bladder cancer (Costa et al., 2014; Brindley et al., 2015). From our perspective, novel therapeutic approaches should focus not only on the elimination of the parasite but also ameliorate the pathologies associated with the infection.

We speculate that a novel therapeutic approach based on drug repurposing and a combination of different active agent might be a valuable tool not only to eliminate the parasite but also ameliorate the infection-associated pathologies. The antioxidants presented interesting and promising biological properties. Antioxidants are considered pharmacological safe agents (Ratman et al., 2006; Sindhi et al., 2013) and might prevent DNA damage (Allam, 2009), block carcinogenesis (Zahid et al., 2001), and present antischistosomal activity by themselves (Gouveia et al., 2018 and 2019a and b). These properties render them as interesting candidates for use against schistosomiasis. Therefore, we propose a novel therapeutic approach based on drug repurposing and a combination of a different class of drugs (anthelmintic and anticancer) with antioxidants (Figure 1).

This novel therapeutic approach was previously evaluated on newly developed schistosomula (NTS) of *S. mansoni* (Gouveia et al, 2019). Here, we undertook its evaluation against adult worms of *S. mansoni* and its effect on oviposition.

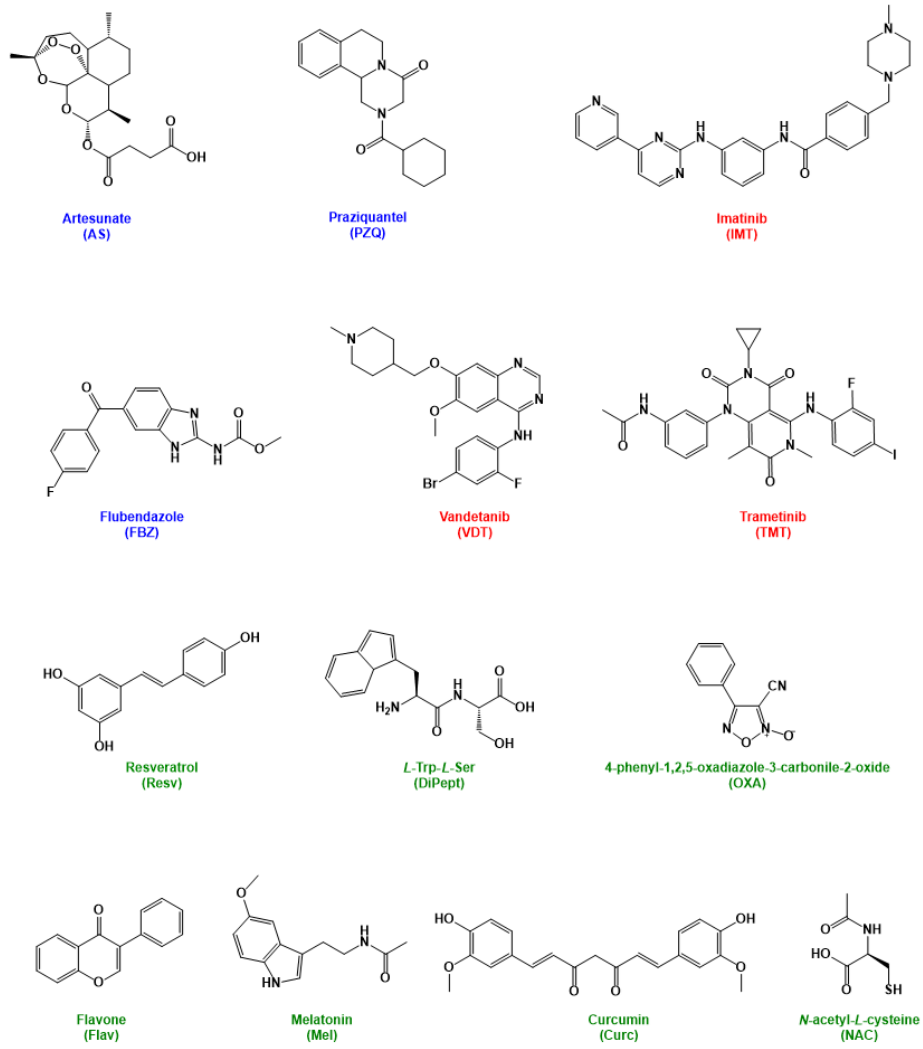

Figure 1. Structures of anthelmintic, anticancer drugs and antioxidant biomolecules evaluated either alone or combined against *S. mansoni* adult worms. The anthelmintic drugs are depicted at blue, anticancer drugs in red and antioxidant biomolecules in green.

2. Material and methods

2.1. *Chemicals and culture media*

Praziquantel (PZQ), 4-phenyl-1,2,5-oxadiazole-3-carbonile-2-oxide (OXA), N-acetylcysteine (NAC), flavona (Flav), flubendazole (FBZ) were purchase from Merck Sigma-Aldrich (Lisboa, Sigma), and resveratrol (Resv) from Santa Cruz Biotechnology (Heidelberg, Germany), artesunate (AS), vandetanib (VDT), curcumin (Curc), and melatonin (Mel) from Cayman Chemical (Ann Arbor, MI, USA) and the dipeptide H-L-tryptophan-L-serine-OH (H-Trp-Ser-OH, DiPept) from Bachem (Bubendorf,

Switzerland). The culture media RPMI 1640 and supplements as penicillin (10,000 U/mL)/streptomycin (10 mg/mL) from Merck Sigma-Aldrich and heat inactivated fetal bovine serum (iFBs) from Lonza (Basel, Switzerland). For in vitro assays, stock solutions of (2-5 mg/mL) were freshly prepared in 100% dimethylsulfoxide (DMSO) (Sigma-Aldrich) and stored at 4ºC. These stock solutions were then diluted in fresh culture media before its addition to the well containing adult worms.

2.2. *Parasites*

The life cycle of the S. mansoni strain is maintained by passage through *Bioamphalaria glabrata* snails and CD1 mice obtained from Center for Vector and Infectious Disease Studies Francisco Cambournac and maintained at animal facility of Public Health Care Dr. Gonçalves Ferreira (INSA-Porto). Female mice CD1 (8 weeks old) were infected with 160-180 cercariae using tail immersion technique (Oliver and Stirewalt, 1952) and after 7-8 weeks days of infection, *S. mansoni* adult worms were recovered under aseptic conditions by perfusion of the livers and mesenteric veins (Smithers and Terry, 1965). The worms were washed in RPMI 1640 medium (Merck Sigma-Aldrich), supplemented with 1% penicillin/streptomycin and 10% bovine fetal serum (iFBS). The experiments were conducted in accordance with the law DL 113/2013 of Portuguese Republic and European Directive 2010/63/UE and were approved by the Ethics of Animal Experiments of INSA-Porto (project no. 04/2018) and Directorate General Food and Veterinary.

2.3. *In vitro antischistosomal activity*

The procedure to evaluate the antischistosomal activity of compounds alone or combined against adult worms was similar to those previously described (Cowan and Keiser, 2015). Briefly, one adult coupled *S. mansoni* worm pair (one female and male) in RPMI 1640 medium (1 ml) were placed in each 48-well plates (Nunclon, Denmark). The screening of test compounds was performed at a concentration of 100 µM and combination at a constant ratio (1:1) at same concentration. All the compounds were prepared as described above and added to RPMI 1640 medium containing the worms after a period of 24h to recover from the eventual stress of perfusion and adaptation to culture medium. The parasites were kept for 72h in a constant temperature incubator at 37ºC in an atmosphere of 5% CO2 and monitored 1, 17, 24, 48 and 72h for motor

activity, mortality and morphological alterations as described elsewhere (Lombardo et al., 2018; Cowan and Keiser, 2015). Adult worms incubated with the highest concentration of DMSO (0.1%) served as negative control. Phenotypic changes were recorded using a light microscope (Nikon Phase Contrast 2, LDW 0.52, Japan). Briefly, the morphological alterations were scored ranging from 0 to 3 (0= all worms dead, 1= minimal activity (severe reduction in motility), severe morphological/tegumental changes, 2= slowed activity (reduced motility, first morphological/tegumental changes, and 3= totally vital, normal active, no morphological changes) (Manneck et al., 2010). Adult worms were considered dead when no movement was observed for at least 2 minutes (Manneck et al., 2010). The percentage of effect was calculated using a previously described equation (1) (Lombardo et al., 2018). All experiments were carried out in duplicate and repeated at least one time.

$$\%\ Effect = 100 - \frac{(Average\ test)*100}{Average\ control} \quad \text{(Eq. 1)}$$

2.4. *Statistical analysis*

The mean and standard deviation were calculated using GraphPad Prism 6 (GraphPad Software, Inc).

3. Results and Discussion

Novel therapeutic approach that not only eliminate the parasite but also could improve and ameliorate the pathologies associated to infection is required against schistosomiasis (Gouveia et al., 2018). Repurposing and combination of drugs with active compounds with a different mode of action might be an effective strategy (Cowan and Keiser, 2015). We hypothesized that combining drugs with antioxidants could enhance not only the efficacy of the drug to eliminate the parasite, but also could improve/prevent sequelae associate with infection (Gouveia et al., 2018). Previously, we evaluate different classes of drugs and antioxidants either alone or combined against newly transformed schistosomula (NTS) of *S. mansoni* and observed that most of the combinations were more active rather than drugs and antioxidant alone (Gouveia

et al., 2019a and b). Herein, we evaluated the efficacy of our novel therapeutic strategy against adult worms of *S. mansoni in vitro*.

3.1. Repurposed drugs and its combination with antioxidants presented interesting antischistosomal activity

As depicted in Figure 2 and Supplementary Figure S1 (see Section VI-Appendix 5), the worms of the control group (RPMI 1640 with 0.1% DMSO) remain viable and without morphological alterations followed 72h post-exposure. Regard to anthelmintic drugs alone, the most active was PZQ with an effect of 86.7% on males and 50.0% on females. PZQ induced the death of male following 24h of exposure while female remains alive but with several morphological alterations after 72h (Supplementary Figure S1 and Figure 2). These results are in consonance with those described in the literature reporting that males are more susceptible to PZQ than females (Vale et al., 2017). In contrast, the males were less susceptible to AS than females as described elsewhere (Utzinger et al., 2007) which were dead follow 48h of exposure. In fact, the effect of AS (20.0% for female and 0.0% for male) was lower than PZQ (Figure 2). Nevertheless, we observed that AS induced the decoupling at 48h of exposure and dead of female at 72h of exposure (Supplementary Figure S1 and Figure 2). Regard to the activity of PZQ and AS against NTS, PZQ induced severe morphological alterations on NTS, yet, most of the larvae remain alive following 72h of exposure while AS induce the death of most larvae (Gouveia et al., 2019a and b). The data obtained for these drugs is in accordance to literature where it is reported that AS is more active against juvenile forms while PZQ is more active against adult worms (Utzinger et al., 2007). The other anthelmintic drug evaluated, FBZ, has been reported that in mice reduce the number of adult parasites of *S. mansoni* (Nessim et al., 2000), however, in this study FBZ had no effect (0.0%, Figure 2) on morphology of males which remained vital and active (Supplementary Figure S1). The female worm seems to be more susceptible to drug presenting slight morphological changes (Figure 2 and Supplementary Figure S1). FBZ is more active against NTS than adult worms even more active than AS or PZQ (Gouveia et al., 2019b). This finding suggested that this repurposed drug is more suitable for administration on the initial stage of infection.

		Score viability					
		1h	17h	24h	48h	72h	Average
	Control	3	3	3	3	3	3.0
	DMSO 0.1% (M)	3	3	3	3	3	3.0
	DMSO 0.1% (F)	2	2	2	2	2	2.0
Only Drugs/Antioxidants	PZQ (M)	1	1	0	0	0	0.4
	PZQ (F)	1	1	1	1	1	1.0
	AS (M)	3	3	3	2	1	2.4
	AS (F)	3	3	3	2	0	2.2
	FBZ (M)	3	3	3	3	3	3.0
	FBZ (F)	3	2	2	2	2	2.2
	TMT	2	1	1	0	0	0.8
	IMT	2	2	1	1	0.5	1.3
	VDT (M)	2	2	2	0	0	1.2
	VDT (F)	2	2	2	1	0	1.4
	Resv	2	2	2	2	2	2.0
	NAC	3	3	3	3	3	3.0
	DiPept	3	3	3	3	3	3.0
	Mel	3	3	3	3	3	3.0
	Flav	2.5	2	2	2	2	2.1
	OXA	1	0	0	0	0	0.2
	Curc	1	0	0	0	0	0.2
Drugs+AntiOx	PZQ+Resv (M)	2	1	0	0	0	0.6
	PZQ+Resv (F)	2	1	1	1	1	1.2
	AS+Resv (M)	3	3	3	2	2	2.6
	AS+Resv (F)	3	3	3	2	0	2.2
	AS+NAC (M)	3	2	2	2	1	2.0
	AS+NAC (F)	3	2	2	2	0	1.8
	AS+DiPept (M)	3	3	2	2	1	2.2
	AS+DiPept (F)	3	3	2	2	0	2.0
	AS+Flav (M)	2	2	1	0.5	0	1.1
	AS+Flav (F)	2	2	1	1	0	1.2
	AS+Mel (M)	3	3	3	1	0.5	2.1
	AS+Mel (F)	3	3	3	0.5	0	1.9
	AS+OXA	1	0	0	0	0	0.2
	AS+Curc	2	0	0	0	0	0.4
	IMT+Flav (M)	1	1	1	1	1	1.0
	IMT+Flav (F)	1	1	0	0	0	0.4
	IMT+Mel (M)	2.5	1	1	0.5	0.5	1.1
	IMT+Mel (F)	2.5	1.5	1	1	0	1.2
	TMT+Mel	2	1	1	0	0	0.8
	TMT+Flav	2	1	1	0	0	0.8
	FBZ+Flav	2	2	2	2	2	2.0
	FBZ+Mel	2	2	2	2	2	2.0
	VDT+Resv	2	1	1	0	0	0.8
	VDT+Flav	1	0.5	0.5	0	0	0.4
AntiOx+AntiOx	Flav+NAC (M)	2	2	2	2	2	2.0
	Flav+NAC (F)	3	3	3	3	3	3.0
	Flav+Mel	2	2	2	2	2	2.0
	Flav+DiPept (M)	2	2	2	2	2	2.0
	Flav+DiPept (F)	3	3	3	3	3	3.0
	Mel+Dipept	3	3	3	3	3	3.0
	Mel+NAC	3	3	3	3	3	3.0
	DiPept+NAC	3	3	3	3	3	3.0
	Curc+OXA	0.5	0	0	0	0	0.1

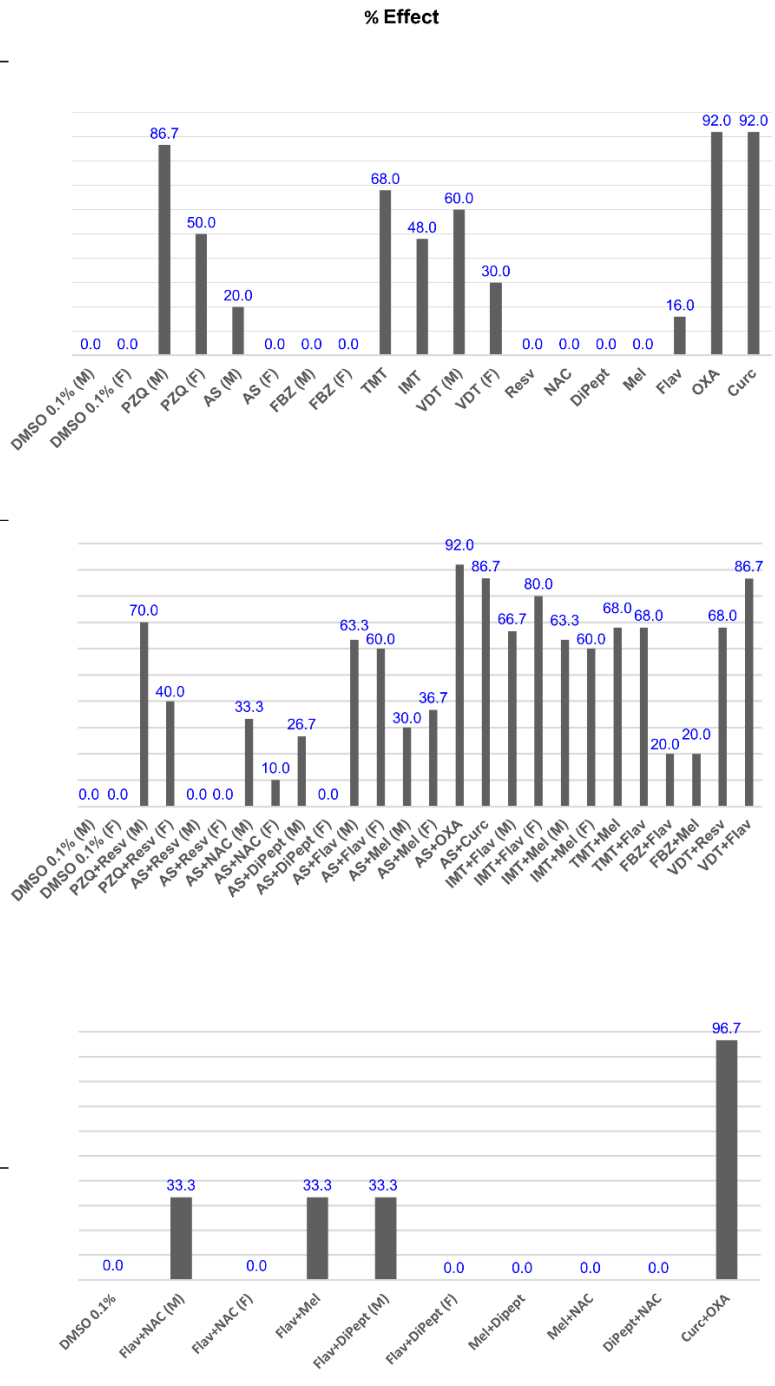

Figure 2. Score viability and graphical representation of percentage of effect (numbers above columns) induced by drugs and antioxidant alone or combined (1:1) at 100 μM against adult worms of *S. mansoni* in vitro. (M-males and F-females).

In general, the antischistosomal activity of anticancer drugs were more pronounce against adult worms than anthelmintic drugs evaluated. From anticancer drugs evaluated TMT was more active (68%), followed by VDT (60.0% for males and 30.0% for females) and IMT (48.0%) (Figure 2). Except for IMT, VDT and TMT induced the dead of both parasites after 48h post-exposure (Figure 2). Regard to worms incubated with VDT was also detectable differences between the sensitivity of male and female (60.0% vs 30.0%), with males being more susceptible than females (Figure 2). IMT caused severe morphological alterations but not the death of parasites (Figure 2), however, lead to a decoupling of worms following 17h post-exposure. Curiously, anticancer drugs also presented an interesting antischistosomal activity against NTS (Gouveia et al., 2019b) counteracting one of the major drawbacks of PZQ. These anticancer drugs are kinases inhibitors and kinases play a pivotal role for different physiological processes namely reproduction and egg production (Grevelding et al., 2018). Most likely, the antischistosomal activity observed could derive from potential inhibition of these enzymes on parasites.

Of antioxidant evaluated, OXA and Curc were highly active leading to the death of both parasites following 17h of exposure which translated in a percentage of effect above 90.0% (Figure 2). OXA and Curc were more active than PZQ itself (Figure 2) like observed against NTS (Gouveia et al., 2019b). Despite the mechanism of action of these two antioxidants are uncertain, some reports attributed activity of OXA to the fact that it could inhibit thioredoxin glutathione reductase (TGR) which is essential for parasite survival (Sayed et al., 2008) while Curc probably could be involved in parasite biochemical processes as ubiquitin-proteasome pathway (Chen et al., 2012). Regard to other antioxidants evaluated, Resv, NAC, DiPept and Mel, did not shown any antischistosomal activity against adult worms similarly to observed for NTS (Gouveia et al., 2019b). The parasites incubate with these antioxidants remained viable without any significant morphological alterations during the assay (Figure 2 and Supplementary Figure S1). Intriguingly, Resv displays a moderate antischistosomal activity (Gouveia 2019b) against NTS while only slight morphological alterations were observed against adult worms (Figure 2 and Supplementary Figure S1) mainly on the females. On the other hand, Flav had a minimal effect (16.0%, Figure 2) causing slight morphological alterations (Figure 2) but not the death of parasites. In these cases, no differences in susceptibilities between males and females were observed. By contrast,

Flav presents moderate antischistosomal activity against NTS (Gouveia et al., 2019b). The mode of action for these two antioxidants remain unknown, however, has been hypothesized that Resv could act on neuromotor activity (Gouveia et al., 2019a and b) while Flav could modulate key cellular enzyme functions (Panache et al., 2016). Further studies are required to understand which the targets of antioxidants and drugs on adult forms of *S. mansoni* are.

Herein, we evaluated if combining antioxidant with drugs enhance their antischistosomal activity. The antischistosomal effect in the combination of drugs with antioxidants was identical to those induced by drugs (e.g. TMT+Mel or TMT+Flav) or antioxidants alone (e.g. AS+OXA and AS+Curc) (Figure 2). Nevertheless, the antischistosomal activity observed in combinations as AS+NAC, AS+DiPept, AS+Flav, AS+Mel, IMT+Flav, FBZ+Flav or Mel, VDT+Resv or Flav, was slightly better than drugs alone (Figure 2). The enhancement of the antischistosomal activity of IMT+Flav and VDT+Resv was more pronounced especially against females (Figure 2). While the combination of VDT+Flav induces a percentage of effect of 86.7%, the drug alone has an effect of 60% for male and 30% for female (Figure 2).

On the other hand, combinations as PZQ+Resv and AS+Resv apparently act as an antagonist, especially against males. The male's worms incubated with these combinations presented a better viability score than those with drug alone. This translate into a higher percentage of effect of drug alone rather than combinations (Figure 2). Interesting, these combinations against NTS were classified as synergistic (Gouveia et al., 2019). These findings could suggest that the combination of the mode of action of PZQ or AS with Resv could be more effective and suitable for NTS than adult worms. Presumably, these combinations are more suitable for the initial stage of infection rather than chronic infection where worms are already fully developed. By contrast, combinations as FBZ+Flav, IMT+Mel or AS+Mel that act as slightly or antagonistic against NTS (Gouveia et al., 2019b), here, enhance the antischistosomal effect in comparison to compounds alone (Figure 2). This reinforces the notion that development stages of parasites have different susceptibilities which suggest that targets are different on NTS and adult worms.

Additionally, the combination of antioxidant and antioxidant was also assessed against adult worms. The antioxidants that presented slight or no antischistosomal activity when evaluated alone were combined (Figure 2). In general, these

combinations did not improve their antischistosomal activity, most of them had 0.0% of the antischistosomal effect (Figure 2). Nevertheless, NAC, DiPept, and Mel combined with Flav display a moderate activity (33.0%, Figure 2) especially against males (e.g. Flav+NAC and Flav+DiPept). These combinations slightly improved the activity of Flav alone (33.0% vs 16.0%, Figure 2). Similarly, the two antioxidants more active (i.e. Curc and OXA) when combined slightly increase their antischistosomal activity against adult worms (Figure 2).

3.2. Oviposition is highly affected after exposure of parasites to antioxidants either alone or in combination with drugs

The pathologies associated with chronic schistosomiasis occurs mainly due to egg deposition that triggers an inflammatory reaction leading to the formation of granuloma in organs where parasite reside (Hussein et al., 2012). The egg production not only induces pathologies-associated to the infection but is also an indicator of worm viability. Thus, oviposition could be an important target for the development of novel approaches for schistosomiasis (Badr et al., 1999). The reproductive capacity of parasites is based on two major criteria: pairing and egg production. The first indicate if the mating process occurs and the latter is an indicator of egg output (Veras et al., 2012). Although some compounds alone or combine did not induce the death of parasites, they could prevent oviposition which could be crucial to counteract the pathologies associated with infection [(Supplementary Table S1, see Section VI-Appendix 5) and Figure 3]. All drugs evaluated against adult worms ceased oviposition either by inducing their death (e.g. PZQ) or by decoupling (e.g. AS). Interestingly, FBZ did not induce severe morphological alterations or decoupling, however, leads to a cessation of the oviposition suggesting that the reproductive system of the female was affected. Indeed, the percentage of antischistosomal effect of FBZ was more pronounced in females rather than males (Figure 2).

Regard to antioxidants OXA, Curc, Flav, and Resv alone also cease the oviposition (Figure 3). In the case of OXA and Curc, this occurs due to the death of parasites. On the other hand, like FBZ, Flav and Resv did not induce severe morphological alterations or dead of parasites (Figure 2), however, they cease the oviposition (Figure 3). The mechanism of action of these antioxidants remains elusive. Nevertheless, based on these findings it is reasonable hypothesized that might be related to the

degenerative process of the female apparatus, at least in vitro. In contrast, the other antioxidants evaluated (DiPept, NAC) did not affect oviposition nor morphological alterations (Figure 2 and 3) reinforcing that they do not present any antischistosomal activity. However, all combinations of drugs with antioxidants also leads to the cessation of oviposition in a similar fashion to observed with compounds alone. Therefore, it is reasonable hypothesized that it could be related to the activity of drugs or antioxidant alone (i.e. Flav and OXA) and not the combination itself.

In combinations of antioxidant and antioxidant, the cessation of oviposition only occurs when Flav was combined with other antioxidants (Figure 3), thus, this might be related to the antischistosomal activity of Flav. The other combinations evaluated (e.g. Mel+DiPept, Mel+NAC or NAC+DiPept) did not affect the oviposition. As mentioned above these antioxidants did not induce any morphological alterations which are in consonance to the fact that parasites continue producing eggs during the assay.

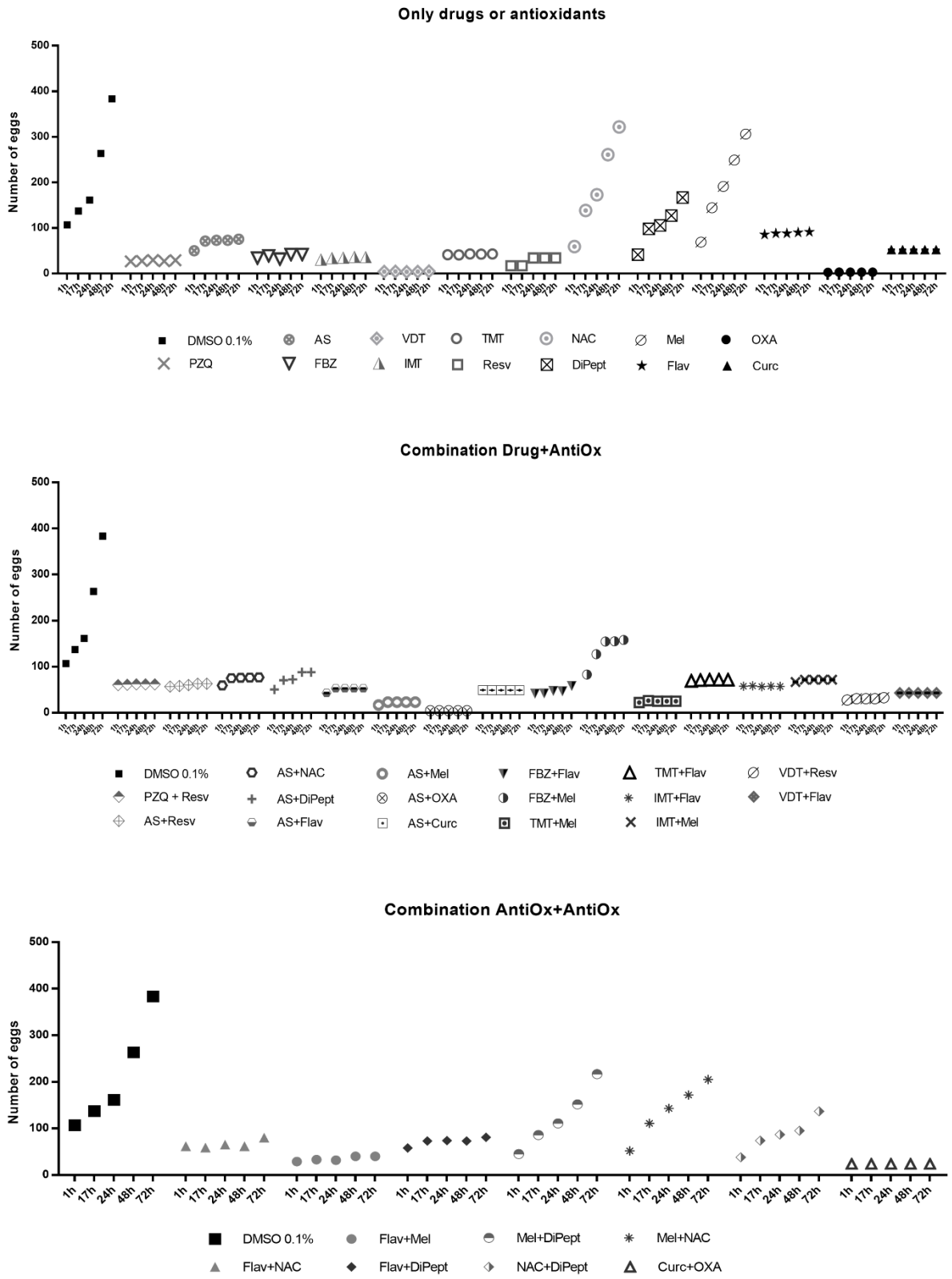

Figure 3. Effect of drugs and antioxidants alone or combine on oviposition. The points represented the number of eggs observed per pair incubated with compounds alone or combine during the assay *in vitro*.

4. Conclusion

To conclude, we report the effect of the different class of drugs and antioxidant biomolecules alone or combine against adult worms of *S. mansoni*. Not only some antioxidants alone (e.g. OXA and Curc) were highly active against adult worms, but also some combinations (e.g. drugs plus antioxidant or antioxidant plus antioxidant) enhance antischistosomal activity against adult worms. The increase of antischistosomal activity might be related to their different mode of action and/or targets on adult's worms. Since eggs are one of the major factors for inflammatory processes related to the parasitic infection (Hussein et al., 2012), and implicated on development of bladder cancer in infection with S. haematobium (Brindley et al., 2015), it might be relevant cease the oviposition. Indeed, the combination of different active agents evaluated during this study achieve the cessation of the oviposition, at least in vitro. Taken together with the previous results with NTS (Gouveia et al., 2019b) and against adult worms, repurposing of drugs as anticancer (or others) might be worthwhile since they were effective against all developmental stages of *S. mansoni* (Gouveia et al., 2019b). Also, due to their biological properties of antioxidants in prevention of DNA damage and blocking cancer initiation process (Allam, 2009; Zhaid et al., 2001), they could play a pivotal role in amelioration of sequelae associated to infection (Gouveia et al., 2018) and even counteract carcinogenesis during infection with helminth parasites, namely *S. haematobium* (Correia da Costa et al., 2014; Brindley et al., 2015) through cessation of oviposition or by prevent the formation of potential parasitic metabolites implicated in carcinogenesis associated to schistosomiasis haematobia. Future studies are required to evaluate these combinations either in vivo in laboratory rodent models and cells lines to understand their effect on amelioration of sequelae and cancer induced by infection of *Schistosoma* species.

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Interest Statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Supplementary information **(see Section VI – Appendix 5)**

Table S1. Oviposition expressed as the mean of number of eggs deposited per worm during in *vitro* assay.

Figure S1. Representative micrographs of adult worms following 72h of exposure to anthelmintic [praziquantel (PZQ), artesunate (AS), flubendazole (FBZ)], anticancer drugs [imatinib (IMT), trametinib (TMT) and vandetanib (VDT)], antioxidants [(4-phenyl-1,2,5-oxadiazole-3-carbonile,2-oxide (OXA), *N*-acetylcysteine (NAC), resveratrol (Resv), flavone (Flav), H-Trp-Ser-OH (DiPept), melatonin (Mel)] alone and combined (1:1) at 100 µM.

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Chapter 7

Inhibition of the formation in vitro of putatively carcinogenic metabolites derived from S. haematobium and O. viverrini by combination of drugs with antioxidants

CHAPTER 7. Inhibition of the formation in vitro of putatively carcinogenic metabolites derived from *S. haematobium* and *O. viverrini* by combination of drugs with antioxidants

Authors: Maria João Gouveia, Verónica Nogueira, Bruno Araújo, Fátima Gärtner, and Nuno Vale

During the research paper presented in this Chapter we evaluate the efficacy of a novel therapeutic strategy to prevent the formation of putative carcinogenic metabolites precursors and DNA adducts. Using the same methodology described on chapter 4, we synthesized metabolites and DNA adduct from starting compounds glycocholic acid and taurochenodeoxycholate sodium in presence of CYP450 *in vitro*. The products of reaction were identified and characterized by liquid chromatography coupled to mass spectrometer (LC-MS/MS). The next phase consisted in evaluating the inhibition of their formation induced by drugs and antioxidants alone or combined in vitro. For that, we incubate the starting compounds, DNA, CYP450, drugs (e.g. AS and PZQ) and antioxidants (e.g. NAC and Resv) either alone or combined and analysed by LC-MS/MS followed 72h of reaction. Drugs and resveratrol alone did not present a significant inhibitory effect while *N*-acetylcysteine inhibited the formation of most metabolite precursors and DNA adducts. The inhibitory effect of drugs seems to be affected by CYP450. This could be counteracted by combine drugs with agent that modulate CYP450 activity as Resv. Indeed, the combinations of drugs plus antioxidants were more effective rather compounds alone, especially AS+Resv which inhibit most of metabolites and DNA adducts. Therefore, the therapeutic strategy might be a valuable tool to prevent initiation of helminth infection-associated carcinogenesis.

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Inhibition of the Formation In Vitro of Putatively Carcinogenic Metabolites Derived from *S. haematobium* and *O. viverrini* by Combination of Drugs with Antioxidants

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Abstract: Infections caused by *Schistosoma haematobium* and *Opisthorchis viverrini* are classified as carcinogenic. Although carcinogenesis might be a multifactorial process, it has been postulated that these helminth produce/excrete oxysterols and estrogen-like metabolites that might act as initiators of their infection-associated carcinogenesis. Current treatment and control of these infections rely on a single drug, praziquantel, that mainly targets the parasites and not the pathologies related to the infection including cancer. Thus, there is a need to search for novel therapeutic alternatives that might include combinations of drugs and drug repurposing. Based on these concepts, we propose a novel therapeutic strategy that combines drugs with molecule antioxidants. We evaluate the efficacy of a novel therapeutic strategy to prevent the formation of putative carcinogenic metabolites precursors and DNA adducts. Firstly, we used a methodology previously established to synthesize metabolites precursors and DNA adducts in the presence of CYP450. Then, we evaluated the inhibition of their formation induced by drugs and antioxidants alone or in combination. Drugs and resveratrol alone did not show a significant inhibitory effect while *N*-acetylcysteine inhibited the formation of most metabolite precursors and DNA adducts. Moreover, the combinations of classical drugs with antioxidants were more effective rather than compounds alone. This strategy might be a valuable tool to prevent the initiation of helminth infection-associated carcinogenesis.

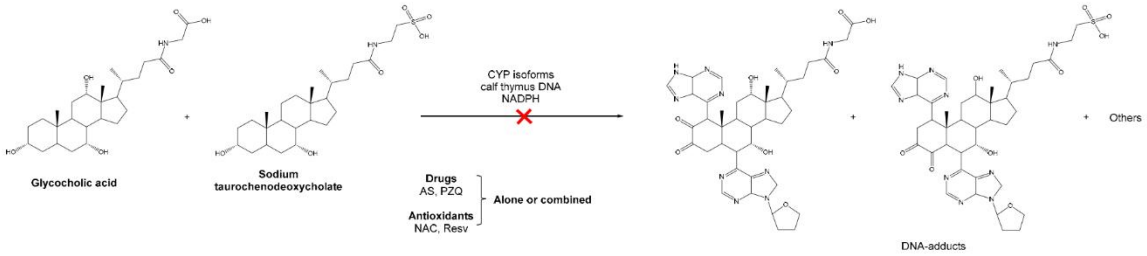
Keywords: helminth infections; carcinogenesis; DNA adducts; CYP enzymes; antioxidants; drug combination; drug repurposing

1. Introduction

Helminths are the most common infectious agents of humans in developing countries affecting about one-third of the world's population [1]. About 20% of cancers in these regions are caused

by infections [1,2], including schistosomiasis, one of the major neglected tropical diseases and opisthorchiasis [3]. Their causative agents, *Schistosoma haematobium* and *Opisthorchis viverrini* are considered as biological carcinogenic agents of group 1, i.e., its infection leads to squamous cell carcinoma (SCC) of the urinary bladder and cholangiocarcinoma (CCA), bile duct cancer [4]. It is estimated that 5000 deaths annually occurred in Northeast Thailand derived from *O. viverrini*-associated cholangiocarcinoma [5,6]. By 2006, the incidence of schistosomiasis haematobia-associated bladder cancer is 3–4 cancer per 100,000 annually but it is plausible to believe that this number is underrated [7]. Notably, case reports from highly endemic regions indicate that patients with schistosomiasis may develop bladder cancer in their first or second decade of life [8]. However, the molecular and cellular mechanism linking these infections with associated cancers remain elusive. Recently, our group indicate that schistosomes and opisthorchiids produce/excrete estrogen- and oxysterol-like metabolites that might react with host DNA leading to its oxidation or forming depurinating DNA adducts. This interaction induces mutations in the genome of adjacent host tissues that could trigger the carcinogenesis. Therefore, the metabolites derived from helminths might be considered as initiators of carcinogenesis [2,9]. Oxysterols and estrogen-like metabolites were identified and characterized through high-performance liquid chromatography coupled with mass spectrometry (LC-MS/MS) in sera, urine from individuals with urogenital schistosomiasis (UGS) and bladder cancer [10–12], and in developmental stages of *S. haematobium* and *O. viverrini* [13]. More recently, similar metabolites and evidence of their interaction with host DNA were identified in biofluids from hamsters experimentally infected with *O. felineus* reinforcing the notion that parasite might also have a potential carcinogenic similar to *O. viverrini* [14]. A pertinent question arises: how are these metabolites derived from helminths formed? Previously our research group performed in vitro assays to observe the interaction of metabolites, similar to those derived from helminths and associated with schistosomiasis and opisthorchiasis-associated cancers, with DNA in presence or absence of CYP450 isoforms [15]. The CYP450 isoforms as CYP1A1, 2E1 and 3A4 are related to the metabolism of chemical carcinogens associated with several human cancers [16]. Also, they are responsible for the metabolism of most drugs [17,18]. Genomic studies identified members of the CYP450 family genes in schistosome and opisthorchiids [19–21].

The current treatment against these helminth diseases relies on a single drug, praziquantel (PZQ) that mainly targets the parasite [22]. PZQ is effective against all forms of schistosome and opisthorchiids and has a safety profile. Nevertheless, the drug has major drawbacks including inefficiency against juvenile forms of parasites, and alone they do not prevent/ameliorate pathologies associate with infection [23]. In addition, there is a legitimate concern of resistance to PZQ due to its extensive use [24,25]. Given that parasites produce putative carcinogenic metabolites, the novel therapeutic strategy should not only target the parasite but also prevent their formation of these helminths derived metabolites and ultimately block carcinogenesis. With this in mind, we proposed a novel therapeutic strategy-based on drug repurposing and rational combination of drugs with antioxidants. These strategies have several advantages such as reducing costs and time of drug development, achieving an additive/synergistic therapeutic effect allowing lower doses and thereby reducing adverse effects and minimizing or delaying the onset of drug resistance [26,27]. Antioxidants presented interesting biological properties which might prevent DNA damage, block carcinogenesis, improve and ameliorate histopathological parameters, and are pharmacological safe agents [28–32], rendering them as interesting candidates to use in therapy against these diseases. Here, we conducted in vitro assays to evaluate if the novel therapeutic strategy counteracts the formation of these helminth derived metabolites. Thus, we used PZQ (anthelmintic), artesunate (AS) as drug repurposed, antioxidants *N*-acetylcysteine (NAC) and resveratrol (Resv) either alone or in combination (Scheme 1). Impressively, not only did antioxidants alone almost completely inhibit the formation of several precursors and DNA adduct in vitro, but when combined with drugs they potentiated their inhibitory activity.



Scheme 1. Schematic representation of the inhibition by drugs and antioxidants, alone or in combination, of the formation of putatively carcinogenic metabolites derived from schistosomes and opisthorchiids and its associated DNA adducts.

2. Results

Previously, our research group demonstrated the ability of the compounds depicted in Scheme 1 to interact with DNA in vitro leading to the formation of DNA adducts. It should be noted that the compounds are similar to those detected in developmental stages of schistosomes and opisthorchiids [15]. Herein, we evaluated the potential inhibition of its formation using the new therapeutic strategy.

2.1. Formation of Metabolites Precursors and DNA Adducts in Presence of CYP450

Due to difficulty in obtained metabolites from extracts of parasites, we selected two commercial compounds similar to metabolites derived from helminths. Previously, we observed that the production of metabolites and DNA-adducts were independent of CYP450 enzymes [15]. In this study, we included the CYP450 isoforms and the reaction mixtures as they may be involved in drug and antioxidant metabolism and consequently affect their activity [17,18]. Considering our previous assay, a period of 72 h of the reaction was selected for this study.

Initially, we performed an assay to identify the metabolites precursors and DNA adducts formed in presence of starting compounds (glycocholic acid and taurochenodeoxycholate sodium) and in the presence or absence of CYP450 isoforms. Then, we compared the LC-MS/MS data obtained for these two samples (data not shown) and selected the exclusive metabolites precursors and their DNA adducts formed in the presence of CYP450 isoforms. From this selection, we obtained a total of 29 compounds in which 17 correspond to precursor metabolites (*m*/*z* 330–500) and 12 to DNA adducts (*m*/*z* 500–770). Their postulated molecular structural is depicted in Figure 1. It is important to note that several of the metabolites and DNA adducts detected in these samples or the oxidized form [M + 16] are already associated with helminth infections caused by *S. haematobium* and *O. viverrini* [10–14].

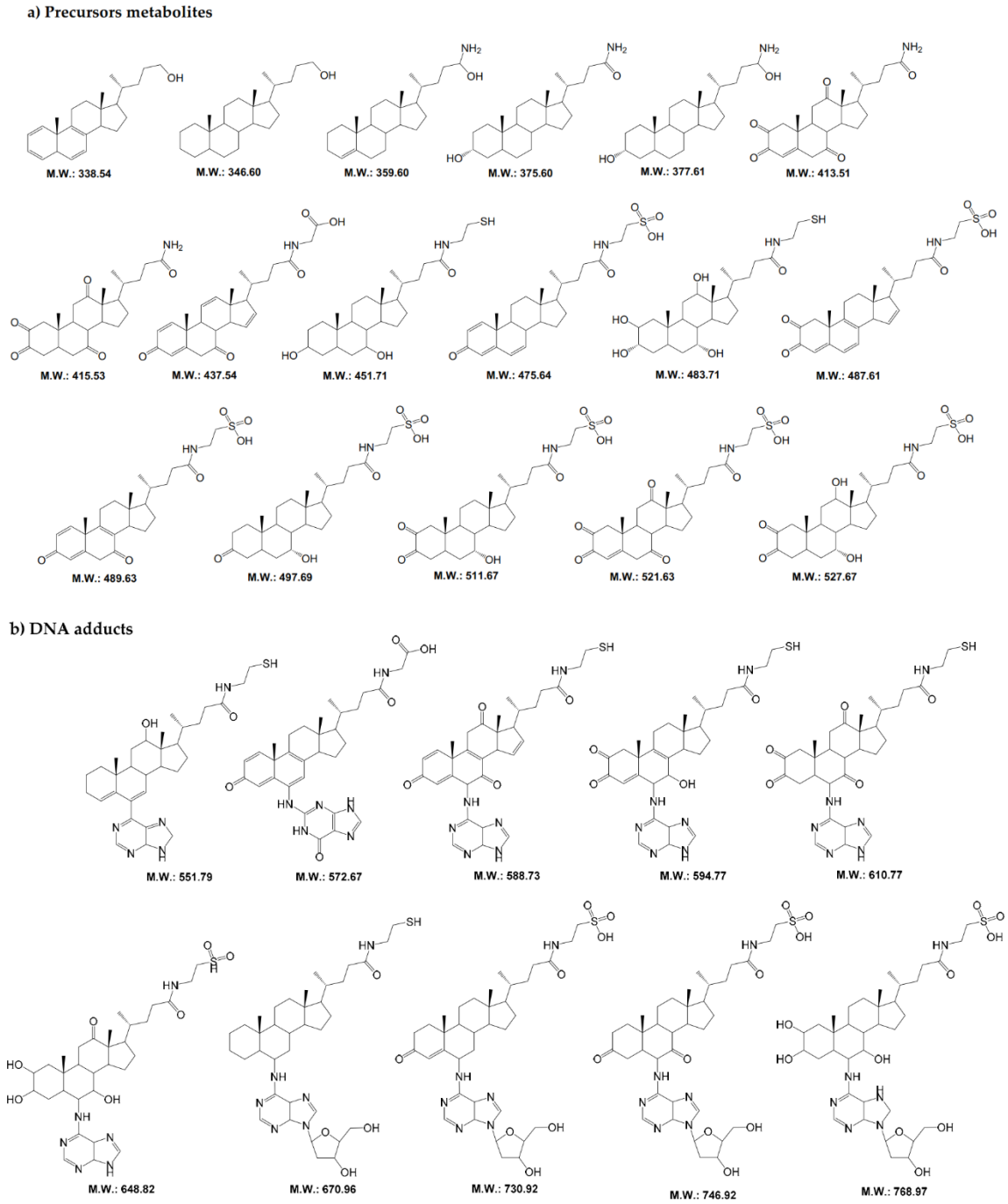


Figure 1. Postulated molecular structures for (**a**) metabolites precursors and (**b**) DNA adducts originated from the reaction of glycocholic acid and taurochenodeoxycholate sodium with calf thymus DNA in vitro in presence of CYP450 isoforms. (M.W., molecular weight).

2.2. Novel Therapeutic Approach Inhibited the Formation of Metabolites Precursors and DNA Adducts.

Following that, we evaluate if the novel therapeutic approach inhibits the formation of metabolites precursors and/or its DNA adducts previously synthesized. For that purpose, reaction mixtures containing starting compounds, CYP450 isoforms, and drugs or antioxidants alone or combined were performed in vitro as described in Section 4. The chromatographic profiles and *m*/*z* obtained by LC-MS/MS are depicted in Supplementary Figure S1. The number of *m*/*z* detected in the combination of drugs with antioxidants was more reduced than drugs and antioxidants alone

(Supplementary Figure S1). The data obtained for all samples were compared with the list of metabolites and DNA adducts detected on control to evaluate the inhibition of their formation (Table 1).

Table 1. Evaluation of inhibition of the formation of precursors metabolites and DNA adducts detected in the control sample by drugs and antioxidants alone and combined. The structures of these *m/z* compounds are represented in Figure 1. The tick and cross indicate the presence (√) or absence (×) of the correspondent metabolite or DNA adduct in the samples analyzed in this study.

	Samples									
m/z	**PZQ**	**AS**	**NAC**	**Resv**	**PZQ + Resv**	**PZQ + NAC**	**AS + Resv**	**AS + NAC**	**PZQ + AS**	**NAC + Resv**
338.90	√	√	√	×	√	√	×	×	×	√
346.87	×	×	√	×	×	×	×	×	√	×
362.93	×	√	×	√	√	×	×	×	×	×
376.86	√	√	×	√	×	×	×	×	×	√
378.90	√	×	√	√	√	×	√	√	√	√
414.90	×	√	×	√	√	×	×	×	×	×
416.91	√	√	×	√	√	×	×	×	√	√
436.88	√	√	×	√	√	×	×	×	√	√
452.85	√	√	×	√	√	×	×	×	×	√
474.83	√	√	×	√	√	×	×	×	√	×
482.20	√	×	×	×	√	√	×	×	√	√
488.31	√	√	×	×	√	×	×	×	√	×
490.79	√	√	×	√	×	×	×	×	×	×
496.81	√	√	×	√	√	√	×	×	√	√
512.79	√	√	×	√	√	×	×	√	×	×
522.29	√	√	×	×	×	√	×	×	×	×
528.84	√	√	×	√	√	×	×	√	×	√
550.83	√	√	×	√	√	×	×	×	√	×
572.79	√	×	√	√	√	√	×	×	×	×
588.79	√	√	√	√	√	√	√	√	√	√
594.78	√	√	×	√	√	√	×	×	√	×
610.77	√	√	×	√	√	√	×	×	×	×
626.81	√	√	×	√	√	×	×	×	×	√
632.83	√	√	×	√	√	√	×	×	×	√
648.81	√	×	√	√	×	×	×	×	√	×
670.79	√	×	×	√	√	×	√	√	√	×
730.81	√	√	×	√	×	√	×	×	√	×
746.78	√	×	×	√	√	√	×	√	√	×
768.70	√	√	×	√	×	×	×	√	√	×

Abbreviations: ***m/z***—ratio mass(m)/charge(z); **PZQ**—praziquantel; **AS**—artesunate; **Resv**—resveratrol; **NAC**—N-acetylcysteine; **PZQ + Resv**—combination of praziquantel plus resveratrol; **PZQ + NAC**—combination of praziquantel plus *N*-acetylcysteine; **AS + Resv**—combination of artesunate plus resveratrol; **AS + NAC**—combination of artesunate plus N-acetylcysteine; **PZQ + AS**—combination praziquantel plus artesunate; **NAC + Resv**—combination of N-acetylcysteine plus resveratrol.

The drugs alone did not significantly inhibit the formation of precursor metabolites or DNA adducts (Table 1). Nevertheless, the inhibitory effect of AS was slightly higher compared to PZQ. AS inhibited the formation of three precursors and four DNA adducts while PZQ only inhibited the formation of three precursors and had no effect on the formation of DNA-adduct (Table 1). Regarding antioxidants alone, NAC was the most potent to inhibit the formation either of metabolites precursors or DNA adducts; it only detected three metabolites precursors (e.g., *m/z* 338.90, 346.87, 378.90) and three DNA adducts (e.g., *m/z* 572.79, 588.79, and 648.81). Resv presented a similar effect as PZQ inhibiting only five metabolites precursors and curiously did not affect the production of DNA adducts (Table 1 and Figure 2).

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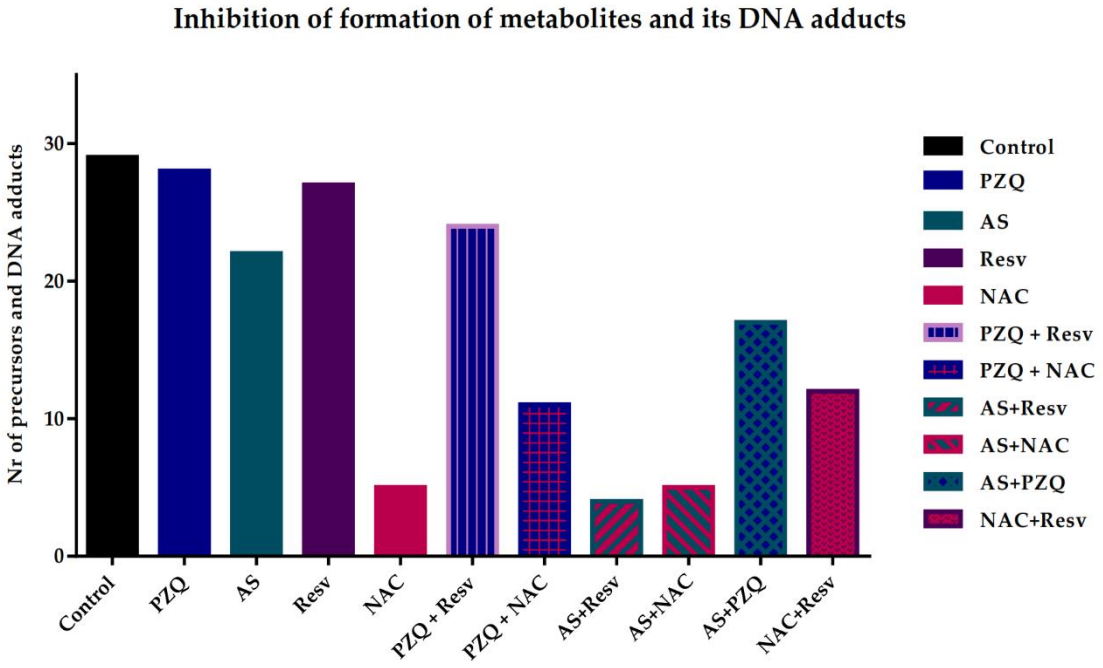

Figure 2. Graphical representation of the number of metabolite precursors and DNA adducts detected in different samples. In general, combination presented better results than compounds alone with AS + Resv inhibiting the formation most of metabolites precursors and DNA adducts. Additionally, antioxidant NAC also prevents the formation of them.

The inhibition of formation metabolites and DNA adducts was more pronounced when drug and antioxidants were combined (Table 1 and Figure 2). Combinations of AS with antioxidants were more effective in inhibiting their formation in comparison to PZQ plus antioxidants (Figure 2). Indeed, AS + Resv resulted in the highest inhibition presenting only three metabolites and DNA adduct detected on control (Table 1). This evidence suggests that the discrete effect of drug and antioxidants alone is potentiated when compounds are combined leading to a potential synergistic effect. In the case of AS + NAC, the inhibitory effect was similar to those observed for NAC alone (7 vs. 6) suggesting that the effect observed in combination derived from antioxidants (Figure 2). The inhibitory effect of PZQ plus antioxidants was slightly better than the drug alone. Of these combinations, PZQ + NAC had a better result in comparison to PZQ + Resv. The combination of PZQ + NAC inhibited the formation of 17 metabolites precursors/DNA adduct while PZQ + Resv only inhibited the formation of 7 compounds. The inhibitory effect observed in PZQ + NAC most likely derived from NAC, nevertheless, in combination the effect was not so pronounced as NAC alone. The combination PZQ + Resv, that inhibited the formation of 7 metabolites precursors or DNA adducts, was slightly better than both compounds alone; PZQ inhibited 7 and Resv 5 (Figure 2).

Additionally, we evaluated the inhibitory effect of the formation of the combinations of a drug plus drug (AS + PZQ), and antioxidant plus antioxidant (Resv + NAC). The combination of AS + PZQ achieved better results than drugs alone inhibiting the formation of a higher number of compounds (13), either precursor's metabolites or DNA adducts, in comparison to the drugs alone (Figure 2). Concerning the combination of NAC + Resv, it was possible to observe a decrease of precursors and DNA adducts more pronounced than Resv alone. However, the inhibitory effect induced by this combination was lower when compared to NAC alone. Nevertheless, the combination NAC + Resv was more efficient than AS + PZQ possibly due to the effect induced by NAC since this antioxidant alone inhibited the formation of most precursors' metabolites and/or DNA adducts (Figure 2).

3. Discussion

Infections caused by carcinogenic agents *O. viverrini* and *S. haematobium* affect millions of people worldwide and are important in terms of mortality and morbidity. Bladder and bile duct cancers are a dire and frequent consequence associated with these infections [33,34]. Probably, the infection-associated with carcinogenesis is a multistep and multifactorial process [35]. Carcinogenesis might undergo a sequence of events that include a pathogenic stimulus, biological or chemical, followed by a chronic inflammation that leads to fibrosis and alters the cellular environment arising a pre-cancerous niche [36]. Regard to opisthorchiasis and schistosomiasis, it was postulated that these pathogens provide biological and chemical stimuli through the production of estrogen and oxysterol-like metabolites that might interact with host DNA triggering a cascade of events that culminate to develop of cancer [2,9]. In our point of view, the therapeutic strategies against these diseases not only should target the parasite but ultimately counteract the formation of these metabolites. Thus, we developed a novel therapeutic strategy based on drug repurposing and the combination of drugs with antioxidants. Previously, this novel strategy demonstrated to be effective against the developmental stages of schistosomes [37,38]. Here, we evaluated the effect of this combination of drugs with antioxidants in inhibition of the formation of metabolites precursors and their DNA adducts.

Recently we explored the generation of some of the metabolites and related DNA-adducts previously identified in the context of opisthorchiasis and schistosomiasis and their infection-associated cancers. In that study, we confirmed the ability of glycocholic acid and taurochenodeoxycholate sodium to interact with DNA leading to the formation of DNA adducts [15]. Their formation might be independent of the parasite CYP450, nonetheless, in the presence of isoforms either metabolites or DNA adducts were also detected. Based on the same methodology, here we synthesized metabolites precursors and DNA adducts of glycocholic acid and taurochenodeoxycholate sodium in presence of isoforms of CYP450. Despite that their formation may be independent of CYP450, this family of enzymes might play a role in the metabolism of drugs and interfere with their activity [17,18]. Through LC-MS/MS analysis we detected several oxidized forms of starting compounds and also their DNA-adducts that were originated during the reaction. The metabolites precursors and their DNA adducts detected here were similar to those observed in our previous work [15].

Impressively, drugs and antioxidants alone, and especially when combined, lead to a reduction of formation of either metabolites precursors or DNA adducts. In fact, it seems that the metabolism of drugs by CYP isoforms altered the drug activity. Among the drugs evaluated, PZQ achieved a lower reduction in their formation which might be related to the fact that PZQ is extensively metabolized by CYP 3A4 resulting in numerous mono- and dehydroxylated derivates [39]. Probably, these PZQ metabolites are less reactive and thus do not prevent the formation of the metabolite precursors and/or DNA adducts. In comparison to PZQ, AS leads to a more pronounced reduction of their formation. The drug is rapidly metabolized to its active form dihydroartemisinin through cleavage of hemisuccinate ester-linked to artemisinin [40]. This suggests that dihydroartemisinin might prevent the formation of some metabolites and/or DNA adducts. However, this metabolite of AS is also metabolized by CYP3A4 to an inactive form [40]. Thus, it is reasonably hypothesized that the conversion of AS to its inactive form reduces the drug capacity to prevent the formation of metabolites and/or precursors of DNA. This might explain why AS is capable of reducing their formation but not significantly. Regarding antioxidants alone, NAC presented the most pronounced inhibitory effect leading to almost complete inhibition of the formation of metabolites precursors/DNA adducts. In other studies, it was already demonstrated that NAC and Resv were able to inhibit the formation of catechol estrogen quinone (CEQ)-DNA [41]. The inhibition of the reaction between precursors metabolites and DNA by the action of these antioxidants might be related to the fact that these antioxidants react with electrophilic compounds, e.g., CEQ, preventing their reaction with DNA, and consequently inhibited the formation of DNA adducts [29,30,41]. Also, NAC reduces semiquinones to their catechol forms and indirectly prevents the formation of DNA adducts [29]. Similar to NAC, Resv also has several important characteristics in the context of carcinogenesis prevention. In studies using cell

lines, Resv was demonstrated to be a quinone reductase (NQO1) inducer, and also is responsible for reducing the electrophilic compounds CEQ in its catechol form, modulating the activity of CYP1A1 that is responsible for catalyzing the oxidation of estrogen to catechol forms [30]. Curiously, in this study, Resv alone did not affect the production of metabolites precursors and its DNA adducts.

Generally, the inhibitory effect was more pronounced when drugs were combined with antioxidants. The combination that achieved better results was AS + Resv which might be related to the fact that Resv could inhibit the activity of isoform CYP3A4 [42]. Resv might block the metabolism of AS or dihydroartemisinin maintain its reactivity, thus, the drug can interact with metabolites precursors and inhibited the formation of DNA adducts. Indeed, it was possible to detect the active metabolite of AS, dihydroartemisinin ([M + H] 284.33, Supplementary Figure S1), in the analysis of a sample from AS + Resv by LC-MS/MS. The combination of PZQ + Resv achieved better results than compounds alone which might be explained similarly to AS + Resv. Regarding the combination of PZQ or AS with NAC, the inhibitory effect might derive from NAC. Nevertheless, the inhibition of formation induced by the combination was lower than NAC alone, which might suggest that NAC might interact with the parental drug, with potentiation of activity but decreasing the antioxidant power.

Interestingly the combinations of drug + drug and antioxidant + antioxidant were not as effective as combinations of drugs with antioxidants or NAC alone. Nevertheless, for AS + PZQ the reduction of metabolites/DNA adducts was more pronounced than compounds alone. These two drugs (at the same time) can be metabolized by the same enzyme, and can occur in competition and, so that, lead to a lower degree of metabolism of PZQ and AS. Thus, there is more availability of drugs to interact with the starting compounds and inhibit the formation of precursor metabolites or DNA adducts. The combination NAC + Resv demonstrated a better inhibitory effect than AS + PZQ which might be related to NAC activity. Nevertheless, NAC + Resv had better activity than Resv alone but not NAC alone suggesting the environment in which biomolecules antioxidants are might influence their antioxidant activity.

To conclude, we demonstrated that antioxidants such as NAC reduced the formation in vitro of most metabolites precursors and their DNA adducts that might be putatively carcinogenic. Despite this, the drugs alone did not induce a considerable reduction, but when combined with antioxidants increased its inhibitory effect. Thus, the novel therapeutic strategy should be pursued since it might be valuable to prevent the *O. viverrini* and *S. haematobium* infections-associated with carcinogenesis by counteracting the formation of putative carcinogenic metabolites derived from helminths. Future studies using informative cell lines should be undertaken not only to assess the carcinogenic effect of these metabolites but also to evaluate the effect of the new therapeutic strategy on inhibiting their formation in the cellular environment and its effect in vitro and in vivo.

4. Materials and Methods

4.1. Reagents and Material

Acetonitrile (ACN) and formic acid (HF), HPLC grade, were obtained from Merck (Darmstadt, Germany). Glycocholic acid hydrated (G2878-100MG), praziquantel (P4668-1G), calf thymus DNA (D1501-100MG), nicotinamide adenine dinucleotide phosphate (NADPH, N7506-25MG), dimethyl sulfoxide (DMSO, D-5879) and CYPExpress™ 1A1 (MTOXCE1A1-250MG), 2E1 (MTOXCE2E1-250MG) and 3A4 (MTOXCE3A4-250MG) were purchased from Sigma/Merck (Sintra, Portugal). Taurochenodeoxycholate sodium (20275) and artesunate (AS, 11817) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Resveratrol (sc-200808) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Absolute ethanol 90% was purchased from Panreac.

4.2. Formation of Precursor Metabolites and DNA Adducts through Their Interaction with DNA In Vitro

The formation of precursors metabolites and DNA in vitro were performed as previously described [15]. Briefly, stock solutions of compounds glycocholic acid and taurochenodeoxycholate

sodium were prepared in 100% DMSO. Then, the compounds at final concentration of 100 µM were incubated with CYP isoforms, CYP1A1, CYP2E1, CYP3A4 (0.2 µM) in the presence of 1.4 µM of NADPH and calf thymus DNA (3 mM) in 67 mM Na-K phosphate buffer (pH 7.2) in a total volume of 200 µL in 96 wells flat bottom plate (Nunclon, Roskilde, Denmark) at 37 °C for 72 h. Aliquots were collected following 72 h after the onset of the reaction. Afterwards, the reaction was ended following the addition of two volumes of cold absolute EtOH to precipitate DNA, which was recovered by centrifugation. Thereafter, 20 µL of the supernatant was subjected to analysis by liquid chromatography coupled to mass spectrometry (LC-MS/MS).

4.3. Inhibition of Formation of Metabolites Precursores and DNA Adducts by Novel Therapeutic Approach

The stock solutions of PZQ, AS, NAC, and Resv were prepared in 100% DMSO. The compounds at a final concentration of 50 µM were incubated with glycocholic acid, taurochenodeoxycholate sodium, CYP isoforms, calf thymus DNA in 67 mM Na-K phosphate buffer (pH 7.2) in a total volume of 200 µL at 37 °C for 72 h, as described above. When combined, the drugs and antioxidants were incubated at a constant ratio 1:1 with the same concentration of 50 µM. The constitution of each mixture reaction is depicted in Table 2. Aliquots were collected 72 h. The reactions were stopped through addition by EtOH, centrifugated, and analyzed as described above.

Table 2. Constituents of the reaction mixtures used for evaluation of inhibition of precursor metabolites and DNA adducts formation by novel therapeutic approach.

	Glycocholic Acid	Taurocheno-Deoxycholate Sodium	NADPH	CYP Isoforms	DNA	PZQ	AS	Resv	NAC
Control	√	√	√	√	√	×	×	×	×
PZQ	√	√	√	√	√	√	×	×	×
AS	√	√	√	√	√	×	√	×	×
Resv	√	√	√	√	√	×	×	√	×
NAC	√	√	√	√	√	×	×	×	√
PZQ + Resv	√	√	√	√	√	√	×	√	×
AS + Resv	√	√	√	√	√	×	√	√	×
PZQ + NAC	√	√	√	√	√	√	×	×	√
AS + NAC	√	√	√	√	√	×	√	×	√
AS + PZQ	√	√	√	√	√	√	√	×	×
NAC + Resv	√	√	√	√	√	×	×	√	√

4.4. Evaluation of Inhibition of Precursores and DNA Adducts Formation by Liquid Chromatography Coupled with Mass Spectrometry (LC-MS/MS) In Vitro

Detection and identification of metabolites and related DNA-adducts by LC-MS/MS were conducted using LTQ Orbitrap XL mass spectrometer (Thermo Fischer Scientific, Bremen, Germany), fitted with an ultraviolet (UV) photodiode assay (PDA) detector as described elsewhere [15]. Briefly, the analysis of aliquots was undertaken by a single injection of 20 µL with an ACE Equivalence 5 C_{18} (75 mm × 3 mm internal diameter) column. The mobile phase consisted of 1% HF in water (A)/CAN (B) mixtures. Elution undergoes at a flow rate of 0.5 mL/min. Eluates were monitored for 8 min, run with mobile phase gradient started with 80% A and 20% B. Then, B was increased linearly to 55% B and 45% A over 5 min and returned to the starting point 5 to 8 min and equilibrated for one minute. Data were collected in positive electrospray ionization (ESI). The capillary voltage of the ESI was 28 kV, and with a temperature of 310 °C. Flow rates of the sheath gas and auxiliary gas (N_2) were set to 40 and 10 (arbitrary units as provided by software settings), respectively, and the gas temperature was 275 °C.

Supplementary Materials: The following are available online, Figure S1: Mass spectra and *m/z* obtained for different samples analyzed by LC-MS/MS.

Author Contributions: Conceptualization, N.V. and M.J.G.; methodology, M.J.G.; V.N.; B.A.; formal analysis, M.J.G.; F.G. and N.V.; investigation, M.J.G.; V.N. and B.A.; resources, M.J.G. and N.V.; writing—original draft preparation, M.J.G.; writing—review and editing, F.G. and N.V.; supervision, M.J.G.; N.V. and F.G.; project administration, N.V.; funding acquisition, N.V.

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Section IV

Discussion

IV. Discussion

1. General discussion

More than 20% of cancers in developing countries are caused by infectious agents. Among these agents are *Schistosoma haematobium* and *Opisthorchis viverrini* that are considered carcinogenic (IARC, 2012). In endemic areas, epidemiological studies related their infection with development of squamous cell carcinoma (SCC) of bladder and CCA, respectively. However, the molecular/cellular mechanism linking these infections with cancer remain elusive. What is the role of helminths in infection-associated carcinogenesis? The carcinogenesis is multistep and multifactorial process (Sripa et al., 2010; Honeycutt et al., 2014), but it is plausible assume that helminths have a role in development of associated cancers either their interaction host-parasite and/or production of metabolites that are putatively carcinogenic. It has been postulated that estrogen and/or oxysterol-like metabolites in form of catechol derived from helminths and DNA adducts have been implicated, at least in part, as initiators of carcinogenesis promoted by these parasites (Correia da Costa et al., 2014; Brindley et al., 2015).

In order to provide deeper insights of UGS-associated cancer, 40 urine from Angolans with UGS, half of who also presented UGS-associated SCC and/or urothelial cell carcinoma were analysed by LC-MS/MS (Section III-Chapter 1). The analysis revealed the presence of numerous estrogen metabolites in the urine of the study participants which seven of these molecules were specifically identified in UGS cases but notably were not described in the recently reported metabolome of urine from healthy humans (Bouatra et al., 2013). The schistosome infection-associated metabolites included catechol estrogen quinones that are potentially reactive with host DNA. Indeed, evidence of this interaction were also observed supported by the presence of DNA adducts in urine. Additionally, evidence of DNA oxidation was also detected in the form of molecules derived from 8-oxodG, a major chromosomal lesion caused by DNA oxidation (Salim et al., 2008; Na et al., 2011). The presence of derivatives from 8-oxodG in the urine of individuals with UGS, supports the notion that metabolites of estrogen derived from schistosome might induce lesions in host cell chromosomes (Botelho et al., 2011). Previously, indicators of oxidative stress were

already detected in soluble lysates of the adult developmental stages of *S. haematobium* (Botelho et al., 2010, 2013), and this mechanism is likely involved in induction of SCC during UGS (Salim et al., 2008). Indeed, there is increasing evidence that endogenous DNA damage is a major etiological factor in human cancer (Salim et al., 2008). In other studies, the correlation between UGS and increase of levels of oxidative stress accompanied by continuous DNA damage and repair in urothelial carcinomas has been observed repeatedly (Dedon and Tannenbaum, 2004; Botelho et al., 2013; Ohinishi et al., 2013). Evidence indicate that UGS-associated SCC might arise through a progressive accumulation of genetic changes in epithelial cells. Interestingly, the findings obtained revealed the presence in urine of molecules known from adult worms and eggs of *S. haematobium* (Botelho et al., 2013) which were considered as carcinogenic chemicals that might interact with host DNA. The carcinogenic effect of this estrogen-DNA adducts mediated pathway underlying schistosomal-promoted damage to host genes which could partially explain the link between UGS and SCC of the bladder. In previous studies using cell lines, Chinese Ovary Hamster and HCV29, demonstrated that the co-culture of eggs with cells or exposure to parasite antigens, the cells displayed the “hallmarks of cancer” (Botelho et al., 2009; Nacif-Pimenta et al., 2019). Probably, this might be related to estrogen-like metabolites presented in eggs that are release and induce these cellular responses. Are these urinary estrogen-like metabolites biomarkers of UGS-associated cancer? Biomarkers would be informative in early detection and prognosis of malignancy induced by UGS, and the metabolites detected in this study might be a promising candidate. Nevertheless, it is required a validation in a larger number of individuals with SCC-associated UGS.

Previously, our research group detected several oxysterol-like metabolites in developmental stage of *O. viverrini* that might be carcinogenic in a similar fashion to estrogen-like metabolites (Vale et al., 2013). The fact that its relative *O. felineus* is not considered carcinogenic (IARC, 2012) has raised the question whether these metabolites would also be present in parasite and about its potential carcinogenic. In order to address this question, we undertook not only LC-MS/MS analysis of developmental stages of *O. felineus* but also biological samples from hamster infected with *O. felineus* (Section III-Chapter 2). The results demonstrated several oxysterol-like metabolites in the egg and adult stage of adult *O. felineus* that exhibited marked

similarity to oxysterol-like molecules known from *O. viverrini*. Additionally, it was possible observed evidence of interaction of metabolites derived from helminth with host DNA leading to DNA adducts detected in sera, urine and especially in bile from infected hamsters. The presence of these adducts provides cogent indirect evidence of both mutagenic and carcinogenic potential of infection with *O. felineus*. Therefore, it is reasonable hypothesized that liver fluke-derived metabolites might directly damage the nuclear DNA of cholangiocytes. On other hand, histopathological studies of liver sections from infected hamsters revealed inflammation with severe periductal fibrosis, portal area enlargement and changes in the epithelium of the biliary tract characterized as biliary intraepithelial neoplasia (BilIN) from different grades. The liver sections presented unequivocally BilIN-1 and BilIN-2 and importantly, BilIN-3 that is considered a pre-cancerous niche (Zen et al., 2007). Formerly, the role of liver flukes has been assigned as a tumor cell growth promoter, whereas the role of NDMA is a mutagenic cancer inducer (Thamavit et al. 1978; Maksimova et al., 2016). The data obtained in Chapter 2 indicate that precancerous occurs observed in hamsters is independent of exposure to an exogenous carcinogen such as NDMA. The consonance biochemical and histopathological changes strongly suggested that infection with *O. felineus* is associated with intracellular mechanisms that eventually trigger neoplastic transformation of cholangiocytes and promote biliary carcinogenesis, at least in rodent model. These findings support earlier observations that *O. felineus* might induce CCA in hamsters (Maksimova et al., 2017). It is reasonable to speculate that the pathobiology of *O. felineus* and *O. viverrini* infections are similar (Lvova et al., 2012). We posit that carcinogenesis associated with *O. felineus* mimics malignant transformation induced by infection with *O. viverrini*. Given these findings, the role for liver flukes is not restricted only to promotion of chronic inflammation, establishment of conditions favourable to the promotion and proliferation of incipient cancer cells, and tumor growth, but now can be seen to also include mutagenesis capable of initiation of the malignant transformation. The accumulation of evidence demonstrating the carcinogenic potential of *O. felineus* suggest that its classification might require revision (Pakharukova et al., 2019). Recently, a comparative LC-MS/MS study of soluble extracts from liver fluke *Fasciola hepatica* adult worms (a non-carcinogenic agent) from bile ducts of cattle with *O. viverrini* and *O. felineus* from experimentally-infected hamsters (Section VI - Appendix 7) reveal clear differences in regard the presence of oxysterols in *Fasciola hepatica* or *Opisthorchis* spp.. The oxysterol-like

metabolites were minor components of the extract from *F. hepatica* in contrast to their abundance and diversity in *Opisthorchis* species. We posit that the smaller number of oxysterols-like metabolites in *Fasciola* compared to *Opisthorchis* species might partially explain why definitive carcinogenic potential has not been ascribed to ruminant or human fascioliasis. On other hand, the presence of abundant oxysterols in the *Opisthorchis* liver flukes support the notion that they might implicate on liver fluke infection-induced biliary malignancy.

The next step of our work was understanding how the metabolites associated with helminth infections were formed (Section III – Chapter 3). Recently, we proposed a route to their formation implying the family of CYP450 enzymes (see Section VI-Appendix 1). The CYP450 isoforms are responsible for formation of estrogen and subsequent metabolism and also are involved in the generation of most chemical carcinogens that induce genotoxicity and DNA damage (Hammons et al., 1997; Guengerich and Shimada, 1998; Nebert and Dalton, 2006). Therefore, we performed enzymatic assays *in vitro* using commercial starting compounds, glycocholic acid and taurochenodeoxycholate sodium, similar to those detected and associated with UGS and opisthorchiasis and incubate them with DNA in presence or absence of CYP isoforms (CYP1A1, CYP2E1, CYP3A4). The aim was investigating the role of these enzymes in activation and/or oxidation of parent compounds and formation of DNA adducts. Through these *in vitro* assays we confirmed the ability of these compounds to interact with DNA leading to the formation of DNA adducts. Although CYP450 isoforms might be involved in formation of some metabolites, apparently, they were not crucial during the metabolization of these compounds to form DNA-adducts, since they were detected in aliquots that not contained isoforms. Thus, it is reasonable hypothesized that oxidized and DNA forms detected were probably generated by non-enzymatic autoxidation process (Russel, 2000; Björkhem, 2002). Given that some of oxidized forms of metabolites were detected in aliquots that presents CYP450 isoforms, it is not reasonable rule out that CYP450 might play a role in their oxidation and formation (Furge and Guengerich, 2006). Notably, the metabolites and DNA adducts previously described (Botelho et al., 2010, 2013; Vale et al., 2013), reported on Section III-Chapters 1 and 2, and synthesized during this *in vitro* assay, were generated in absence of CYP450 isoforms. This evidence suggest that the formation of these metabolites also might be independent of the parasite CYP450. Importantly, the

findings observed in this assay supported the previous postulate since we have shown that metabolites derived from glycocholic acid and taurochenodeoxycholate sodium are able to interact with DNA leading to formation of DNA adducts, at least *in vitro*.

Despite several control strategies to block their transmission have been developed including improvements in sanitation, use of molluskicides, health education, among others (Jongsuksuntigul and Imsomboon, 2003; Secor, 2014), and mass drug administration programs, opisthorchiasis and UGS remain a public health problem in endemic countries (Colley et al., 2014; Ogorodova et al., 2015). The current therapy against these helminth infections is based on a single drug, PZQ (WHO, 2006). Is current therapy enough to combat the dire complications associated with these helminth infections? Despite its safety and efficacy, PZQ alone do not counteract pathologies associated with infection including the development of cancer (reviewed in Section VI-Appendix 2). To improve the current therapy for these infections and taken into consideration the evidence presented in Chapters 1 and 2, we hypothesize about developing a novel therapeutic approach with dual mode of action: not only target the parasite but might also suppress the formation of metabolites derived from parasites. This could lead to improvements in infection-associated pathologies and ultimately counteract the development of cancer. With these in mind we proposed a novel therapeutic approach based on drug repurposing and combination with antioxidants. Why do we select antioxidant biomolecules to use in our novel therapeutic approach? Their biological properties might be valuable not only to enhance anthelmintic activity but also block the carcinogenesis through inhibition of formation of putatively carcinogenic metabolites derived from helminths. Due to difficult of *S. haematobium* grow in rodents (Doenhoff et al., 2009), the effect of novel therapeutic approach in elimination of parasite was evaluated in *S. mansoni* that are the most widely schistosome model (Keiser, 2010).

To evaluate the effect of novel therapeutic approach in elimination of parasite we established a mechanical transformation of *S. mansoni* cercariae into NTS and optimize their culture conditions (Section III-Chapter 4). After that, drug sensitivity screenings were undertaken using an inverted and automated microscope combined with fluorometric-based methods. Comparing the antischistosomal activity of drugs alone, AS was more active than PZQ. This finding is consistent with earlier findings that showed that AS is more effective against larval stage (Utzinger et al., 2007).

Regard to antioxidant alone, Resv demonstrated a moderate activity against NTS. Nevertheless, when Resv was combined with PZQ or AS enhance their antischistosomal activity and induce a moderate and marked synergistic effect (Section III-Chapter 4). The synergy in antischistosomal action might result from increasing the action on anthelmintic drugs target or acting concomitantly on different targets (Araújo et al., 2008). This evidence was supported not only by optical microscopic but also by transmission electron microscopy observations. The ultrastructural analysis demonstrated that NTS treated with AS+Resv suffered extensive and severe damage in comparison to controls and NTS treated with AS or Resv alone. The tegumental and subtegumental regions of these NTS showed severe alterations including disruption of tegument, extensive lysis of subtegumental regions with the presence of numerous vacuoles with diverse sizes, and loss of the basal membrane. Nonetheless, NTS treated with AS alone also showed ultrastructural alterations, including loss of integrity of the matrix, and presence of vesicles on the tegument and some tegument disorder of larvae treated with Resv. By contrast, the controls presented regular morphology. Given these observations, the target of AS, which activity is potentiated when combined with Resv, might be the tegument of NTS leading to its disruption and extensive lysis of subtegumental regions. This is important, since the tegumental damage might lead to disappearance of the immunological camouflage of the parasite which, in turn, would expose immunogens and immunogenic epitopes (Shaw and Erasmus, 1987; Xiao et al., 2002; Xavier et al., 2014) and might become vulnerable to host attack *in vivo*. The parasite tegument represents the frontline interface between host and parasite playing a pivotal role in defence of host immune attack. Also, it has essential secretory and nutrient absorption functions (Halton, 1997). The tegument disruption induced by AS combined with Resv *in vitro* might be directly linked to NTS death since following tegument damage parasites lost the support for its nutrition. On other hand, combination of NAC with AS or PZQ did not translate into a synergistic effect. In fact, NAC alone did not present antischistosomal activity. The lack of antischistosomal activity of NAC alone was consistent to observed in other report (Aires et al., 2012).

Followed these encouraging findings we extend the study and evaluated other anthelmintic flubendazole (FBZ), anticancer drugs including imatinib (IMT), vandetanib (VDT) and trametinib (TMT), and several antioxidants against larval stage and adult

worm of *S. mansoni* (Section III-Chapter 5 and 6). The findings indicated that not only do some antioxidants (e.g. Mel, Resv or Flav) improve anthelmintic activity, but they exhibited activity *per se*, leading to high mortality of NTS and adult worms after exposure. The most effective antioxidants against both developmental stages were 4-phenyl-1,2,5-oxadiazole-3-carbonile-2-oxide (OXA) and curcumin (Curc). The mechanism of action of these antioxidants remain uncertain. However, it has been hypothesized that Curc might be involved in parasite biochemical processes with ubiquitin-proteasome pathway might be a possible target. Regard to OXA, its activity might be related to inhibition of thioredoxin glutathione reductase (TGR) that plays an important role for parasite survival (Sayed et al., 2008). The inhibition of TGR might lead to the inactivation of both thioredoxin and glutathione-base defences and the accumulation of ROS and RNS species (Sayed et al., 2008). Antioxidants including flavone (Flav), melatonin (Mel), dipeptide (H-*L*-tryptophan-*L*-serine-OH, DiPept) and *N*-acetylcysteine (NAC) alone demonstrated slight or did not present activity against developmental stages of *S. mansoni*. Nevertheless, the antioxidants might be useful to ameliorate the infections-associated pathologies. For example, in other studies it was demonstrated that Resv and NAC improve oxidative stress, organ dysfunction and restore of enzymatic activity in animal model of schistosomiasis (Soliman et al., 2017). All drugs repurposed presented interesting results against larvae or adult worms, especially the anticancer drugs. The anthelmintic activity of anticancer drugs might derive from the fact that they are kinase inhibitors. Schistosome kinases play a pivotal role in different physiological processes as reproduction which is closely associated with egg production (Grevelding et al., 2018). Indeed, when adult worms were exposed to these drugs the oviposition ceased even if the drug did not lead to their death (Section III-Chapter 6). Regard to FBZ, the drug was more active against NTS rather than adult worms in contrast to reported by other studies that reported the decrease of adult worms recovered from infected animals (Nessim et al., 2000; William et al., 2003). Considering the mechanism of action of FBZ, its activity against NTS might be due to interaction of the drug with microtubules inhibiting the surface membrane maturation of NTS (Wiest et al., 1998; Čáňová, 2017). At least *in vitro*, the drug did not affect the morphology of adult worms which remain alive, however, FBZ ceases the oviposition by female worms. Similar to previously observed in Section III-Chapter 4, the results obtained by combinations of these drugs and antioxidants were promising and demonstrated that even though some antioxidants did not display significant activity

against NTS, that does not necessarily indicate that they were not able to enhance anthelmintic activity of drugs. Several combinations were classified as synergistic against larval stages (e.g. PZQ+Resv or Mel, AS+Resv or NAC, VDT+Flav or Resv) (Section III-Chapter 4 and 5), however, in general, this was not translated into adult worms where the antischistosomal effect of combinations apparent derived from drugs or antioxidants alone (Section III-Chapter 6). The difference might be related to the fact that the target in NTS and adult worms are different. Probably, these combinations might be suitable for initial stage of infection as prophylaxis or for use in regions with intense re-infection levels. The synergism in anthelmintic activity could be a result from increased action against anthelmintic drug targets or by acting concomitantly on discrete targets (Araújo et al., 2008). By contrast, in combinations classified as antagonist (e.g. PZQ+NAC, AS+Mel) the combinations of the compounds might inhibit antischistosomal activity of either the drug or the antioxidant. Other studies also report the different behaviour of combinations (Keiser et al., 2013; Pakharukova et al., 2018). Curiously, the same antioxidant, for example Mel, incubated with different drugs (e.g. PZQ+Mel or AS+Mel) displayed different combination indexes (Section III-Chapter 5) suggesting that activity of the antioxidant varies depending on the drug used for the combination. Additionally, the susceptibility to the drugs and antioxidants alone or in combination differ from male to female worms as reported in the literature for several drugs (Section III-Chapter 6). For example, it is known that females are less susceptible to PZQ or male are less susceptible to AS (Utzinger et al., 2007).

In general, for both development stages, combinations of AS or anticancer drugs plus antioxidants appear to achieve better effect compared to the other combinations. Apparently, AS and anticancer drugs present a better profile for the combination of different active agents preventing the nullification of the activity of one compound over the other. Importantly, drugs and antioxidants either alone or combine not only induce morphological alterations that ultimately culminate with death of parasites but also affects the oviposition. All drugs evaluated against adult worms ceased oviposition either by inducing parasite death or by decoupling. Interestingly, even drugs that did not induce severe morphological alterations or decoupling, however, leads to a cessation of the oviposition suggesting that reproductive system of the female was affected. This feature occurs not only for drugs but also for antioxidants alone (e.g. OXA, Curc, Resv, flavone (Flav)) (Section III-Chapter 6). The cessation of oviposition

is important since eggs release by female worms are responsible for the formation of inflammatory granuloma on target organs, and transmission of these diseases caused by schistosomes or opisthorchiids (Sripa et al., 2011; Hussein et al., 2012; Schwartz and Fallon, 2018). Regard to combinations and its effect in cessation of oviposition the results suggested that they might be related to the effect induced by drug or antioxidant alone and not for the combination itself in a similar fashion to observed against adult worms.

The last step of our work consisted in evaluation of the inhibitory effect in formation of putative carcinogenic metabolites *in vitro* by novel therapeutic approach (Section III-Chapter 7). In this study, not only antioxidants presented interesting results but also the novel therapeutic approach (e.g. drug+antioxidant) presented better activity than compounds alone. Indeed, antioxidant NAC alone inhibit the formation of most of metabolites and DNA adducts *in vitro* while Resv and drugs (i.e. PZQ and AS) alone did not present significant effect. Some studies have been demonstrated that NAC reduces semiquinones to their catechol forms and indirectly prevent the formation of DNA adducts (Zahid et al., 2010, 2011). Also, Resv also has several important characteristics in the context of carcinogenesis prevention: quinone reductase (NQO1) inducer, reduce electrophilic compounds, and modulate the activity of CYP450 enzymes that are responsible for catalyzing the oxidation of estrogen to catechol forms (Zahid et al., 2011). Curiously, Resv alone did not present a significant inhibitory effect in this context. Regard to AS and PZQ, they are rapidly metabolized to inactive forms (Godawska-Matysik and Kieć-Kononowicz, 2006; Morris et al., 2011) which might explain the lower effect observed in comparison to combination of drug+antioxidant. As mentioned, the inhibitory effect was more pronounced when drugs and antioxidants were combined. This increase of effect might be related to ability of Resv in modulate activity of enzymes as CYP3A4 preventing the metabolism of AS and PZQ. The most promising result was achieved by AS+Resv that lead to almost complete inhibition of the formation of metabolites and DNA adducts. Curiously, the combinations of drug+drug and antioxidant+antioxidant were not as effective as drug+antioxidants or NAC alone. Yet, the combination AS+PZQ achieve better inhibitory effect than drugs alone. Both drugs are extensively metabolized by CYP450 isoforms, however, since there are two drugs that can be metabolized by the same enzyme, it might occur a competition and lead to a lower degree of metabolism of PZQ and AS. Thus, there is

more availability of drugs to interact with the starting compounds and inhibit the formation of precursor metabolites or DNA adducts.

We believe that novel therapeutic approach with dual complementary active agents developed during this thesis is expected to come successful therapeutic tools. Ideally, the data obtained should be use for translational research in order to improve life quality and health of populations of endemic areas. Furthermore, a better knowledge of the underlying molecular profile of parasite and host will facilitate the development of novel therapeutic approach more effective in elimination of parasites and ameliorate/prevent the pathologies associated with infection.

It is our hope that this PhD project draw the attention to these neglected helminth diseases that may be re-emerging in developed countries include in Europe.

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Section V

Conclusions and Future Perspectives

V. Conclusions and future perspectives

1. Main findings and conclusions of the PhD project

The main aims of this doctoral thesis were provided deeper insight of the role of parasite-derived metabolites in the development of cancer associated with infections caused by *S. haematobium* and *Opisthorchis* spp. and developed a novel therapeutic strategy to combat these infections that presented dual mode of action.

Regard to the first aim, we believe that the evidence obtained during this thesis may be helpful to elucidate the mechanism of carcinogenesis associated with these helminth infections. Estrogen metabolites previously described in *S. haematobium* developmental stages (egg and adult worms) and sera from patients with UGS were now identified in urine samples from infected individuals. Additionally, the detection of derivatives from 8-oxoG urinary excretion of UGS patients indicates a putative oxidative action of these metabolites to oxidized DNA. This evidence supports the postulate that estrogen-like metabolites mediated the pathway that lead to development of SCC through induce DNA damage either by the formation DNA adducts or by DNA oxidation. We also demonstrated the potential carcinogenic of *O. felineus* that is not considered carcinogenic but represent a potential public health problem in endemic countries. The parasite not only have oxysterol-like metabolites similar to those described for *O. viverrini* but also evidence point out that they interact with host DNA leading to DNA adducts. Also, histopathological features demonstrated that *O. felineus* may induce a favorable environment for development of pre-cancerous niche, at least in rodent model. Additionally, we demonstrated the ability of similar compounds to those associated for UGS and opisthorchiasis to interact with DNA leading to formation of DNA adducts *in vitro*. These findings support the previous postulate which indicate that metabolites derived from helminths might play a role in initiation of infections-associated carcinogenesis due to DNA damage and open avenues for understanding why some helminths cause cancer.

The novel therapeutic approach with dual mode of action by combining drugs with antioxidants achieved promising results. Not only antioxidants by itself were active against the larval and adult forms of the parasite but also enhance the antischistosomal activity of anthelmintic drugs such as PZQ and AS among others. On other hand, the

novel therapeutic strategy was also effective in inhibiting the formation of metabolites previously associated with schistosomiasis and opisthorchiasis *in vitro*. Thus, we have shown that the combination of agents with distinct mode of action, as drugs and antioxidants, is beneficial in eliminating the parasite, either larval and adult forms, but also in preventing the formation of parasite-derived and putatively carcinogenic metabolites. The combination of active agents with discrete mode actions seems to be effective strategy to improve the therapy against these helmintic infections. Based on results obtained and due to their biological properties, antioxidants are a promising candidate to use in therapy, not only alone but also combined. The results obtained will be important in further studies including *in vivo* assays. We believe that our results may help to improve discussion about novel alternatives to current therapy against these dreadful diseases.

2. Future perspectives

We consider that the results obtained and present in section III of this doctoral thesis are promising enough to encourage further research in area of helminth infections and its associated cancer. Additionally, further investigation is warranted in order to decipher relationships between liver fluke oxysterols and malignancy, including opisthorchiasis felinea in humans. These studies will provide further knowledge of opisthorchiasis-associated carcinogenesis. In future, it will be important perform studies using informative cell lines in order to evaluate the potential carcinogenic of metabolites derived from helminths. On other hand, the effect of novel therapeutic approach should be also evaluated in cell lines, *in vivo* and in other species including *S. haematobium* and *Opisthorchis* spp.. Further studies are required to elucidate the mechanism of drugs and antioxidants alone and how they induce synergism when are combined. Furthermore, it is important understand the targets of different developmental stages that will help to development a more targeted and effective therapy. During this thesis we performed preliminary studies regarding to inhibitory effect in formation of metabolites and DNA adducts by novel therapeutic strategy. Nevertheless, its inhibitory effect should be also evaluated in the cellular environment and *in vivo* to assess its effect on amelioration of infection-associated

pathologies. Exploitation of parasite-derived antigens to use in immunotherapy against these helminth infections should also be considered.

Section VI

Appendix

Appendix 1

The role of estradiol metabolism in urogenital schistosomiasis-induced bladder cancer

Review

Tumor Biology

Tumor Biology
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The role of estradiol metabolism in urogenital schistosomiasis-induced bladder cancer

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Abstract

Urogenital schistosomiasis is a neglected tropical disease that can lead to bladder cancer. How urogenital schistosomiasis induces carcinogenesis remains unclear, although there is evidence that the human blood fluke *Schistosoma haematobium*, the infectious agent of urogenital schistosomiasis, releases estradiol-like metabolites. These kind of compounds have been implicated in other cancers. Aiming for enhanced understanding of the pathogenesis of the urogenital schistosomiasis-induced bladder cancer, here we review, interpret, and discuss findings of estradiol-like metabolites detected in both the parasite and in the human urine during urogenital schistosomiasis. Moreover, we predict pathways and enzymes that are involved in the production of these metabolites emphasizing their potential effects on the dysregulation of the tumor suppressor gene *p53* expression during urogenital schistosomiasis. Enhanced understanding of these potential carcinogens may not only shed light on urogenital schistosomiasis-induced neoplasia of the bladder, but would also facilitate development of interventions and biomarkers for this and other infection-associated cancers at large.

Keywords

Schistosoma haematobium, urogenital schistosomiasis, bladder, squamous cell carcinoma, estrogen-like metabolites, estrogen-DNA adduct

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Urogenital schistosomiasis-induced squamous cell carcinoma of the bladder

Schistosomiasis is a major neglected tropical disease (NTD) and is considered the most important human helminth disease in terms of morbidity and mortality.[1,2] Control strategies have been used to block transmission and reduce the burden of this NTD, including mass targeted chemotherapy, improvements to sanitation, modification of the environment, and use of molluscides.[2,3] Although different approaches to control, prevent, or in some cases even eliminate NTDs exist, like water purification or mass drug administration, for many of them, the tools and implementation strategies available are suboptimal, incomplete, or inadequate to sustain elimination effectors.[1,4] Chemotherapy against flatworm infections, such as schistosomiasis and

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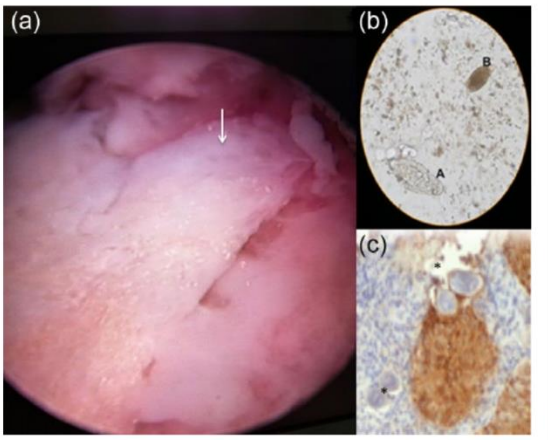


Figure 1. (a) Representative case of urogenital schistosomiasis (UGS)-induced bladder cancer, in a patient residing in an endemic region of UGS in Northern Angola. Arrow indicates the affected area, (b) representative micrographs of eggs of *S. haematobium*, both viable (A) and calcified (B) and (c) Histology of bladder mucosa revealing eggs of *S. haematobium* (asterisks).

opisthorchiasis, is constrained by the limited number of indicated drugs in the market, generally only praziquantel. Emerging drug resistance has been already reported.[5] Yet schistosomiasis remains a major public health problem especially in endemic rural regions of sub-Saharan Africa.[6] Eggs are shed continuously by the *Schistosoma haematobium* adult parasites; these eggs engender chronic, granulomatous inflammation in the bladder wall, leading to severe disease including malignancy (Figure 1).[7] Indeed, bladder cancer is a frequent and grim complication of chronic urogenital schistosomiasis (UGS).[8] The World Health Organization's International Agency of Research Cancer (WHO IARC) categorizes infection with *S. haematobium* as a definitive etiology of cancer, that is, Group 1 carcinogen.[1,9] The severity and frequency of squamous cell carcinoma (SCC) of the bladder are related to the burden and duration of the infection.[8,10] Understanding the mechanisms underlying the *S. haematobium* infection-related carcinogenesis is a key element to define targets for novel control tools-vaccines or new drugs to combat the infection and its consequences.[11]

Estrogen-DNA adduct-mediated pathways may underlie, at least partly, the etiopathogenesis of the UGS-induced bladder cancer.[12–16] Interactions between the estrogen receptor (ER) and total antigen preparation of *S. haematobium* have been described in vitro. Chinese Hamster Ovary (CHO) cells transfected with a construct expressing luciferase driven by the estrogen response element (ERE) were exposed to *S. haematobium* adult worm antigen preparation. Luciferase activity measured in cells exposed to *S. haematobium* antigens was significantly lower than controls, suggesting that the antigen preparation downregulated and repressed the ER activity at least in vitro.[13] Downregulation of ER-alpha and ER-beta may serve to control physiological responses in target tissues.[17] These findings suggest that total antigens dampen the stimulatory response to estradiol.[12] In addition, schistosome may synthesize estrogen-like metabolites, and derived estrogen-DNA adducts that circulate in the blood may be excreted in the urine during UGS.[12,13] The causes and consequences of the formation of these estrogen-derived metabolites, and whether they might promote DNA damage in the bladder urothelium and/or other host tissues remain unclear. Estradiol-like metabolites and novel metabolites of 8-oxo-2′-deoxiguanosine (8-oxodG) were detected in urine of UGS and associated SCC of the bladder cases;[15] 8-oxodG liberated following oxidation of deoxynucleotides, has been implicated in the initiation and/or promotion of inflammation-mediated carcinogenesis, and is indicative of chromosomal damage.[15,18,19] Trapped eggs in bladder tissue induce a distinct immune-mediated granulomatous response that causes local and systemic pathological effects that along with resulting bacterial superinfection and renal dysfunction also can have lethal consequences.[2] Mutations and/or expression dysregulation of *p53* gene have been associated with benign and premalignant lesions and histologically normal mucosa adjacent to the SSC tissue, during UGS-induced carcinogenesis.[20] Determining whether these mutations in *p53* are related to the presence of estrogen-DNA adducts might be instructive.[20]

The tumor suppressor gene *p53*, located on chromosome 17p13, is a frequent target for mutations commonly involved in tumor progression in diverse neoplasias.[21,22] Diverse roles for *p53* have been described, including cell cycle control, DNA repair, activation of apoptosis,[23] inhibition of tumor growth, suppression of cell transformation,[24] and maintenance of genome integrity.[25–27] Loss or mutation of *p53* accelerates tumorigenesis and alters the cell response to agents that damage the DNA.[22] In particular, several mutations in *p53* have been associated with UGS-induced bladder cancer, for example, mutations in the coding region, codon 249, were identified in dysplasic and metaplasic lesions in the urinary bladder or frequently in invasive SCC of the bladder.[19,21,28,29]

Here, we propose a pathway for the formation of these estrogen-like metabolites and describe key enzymes based on recent findings.[12–16] Emphasis is given to the tentative association between these metabolites, dysregulation of *p53* and DNA damage in the target tissue—the urothelium of the urinary bladder-during UGS. Estradiol-like, catechol-like, and/or oxysterol-like metabolites play a role in carcinogenesis as potential initiators of chemical carcinogenesis,[15,30] and several mechanisms may explain their role in the disease. The hormone-mediated mechanism by which the ER is stimulated by some of these metabolites promoting cellular proliferation, increasing the likelihood of errors in DNA replication, has been well studied.

Table 1. Orthologs of enzymes of the steroid biosynthesis pathway encoded in the genomes of *Schistosoma haematobium* and *S. mansoni*.

Enzyme ID	Name	*S. haematobium*		*S. mansoni*	
		E-value	Acc. no.	E-value	Acc. no.
1.14.13.70	Lanosterol 14-alpha demethylase	1.30E−44	A 06730	9.00E−52	Smp 008450.1
1.3.1.70	Delta14-sterol reductase	1.30E−31	A 07198	7.30E−38	Smp 124300.1
1.14.13.159	Vitamin D 25-hydroxylase	5.70E−11	A 00472	–	–
1.3.1.21	7-Dehydrocholesterol reductase	5.80E−24	A 07198	3.90E−33	Smp 124300.1
3.1.1.13	Lysosomal acid lipase/cholesteryl ester hydrolase	1.10E−203	A 04656	2.50E−203	Smp 011000.1
2.3.1.26	Sterol O-acyltransferase	7.10E−257	A 03728	2.10E−273	Smp 134390.1
SMO1	4,4-Dimethyl-9beta,19-cyclopropylesterol-4alpha-methyl oxidase	2.20E−22	A 07158	2.10E−30	Smp 074930.1
CYP51G1	Cytochrome P450, family 51 (sterol 14-demethylase)	1.30E−44	A 06730	9.00E−52	Smp 008450.1
FK	Delta14-sterol reductase	1.30E−31	A 07198	7.30E−38	Smp 124300.1
DWF5	7-Dehydrocholesterol reductase	5.80E−24	A 07198	3.90E−33	Smp 124300.1

Alternatively, estrogen metabolites acting as chemical carcinogens might react covalently with the DNA by redox cycling or by forming an abasic site. Subsequent error-prone repair of the damaged DNA eventually results in chromosomal lesions that initiate carcinogenesis.[14,30–33]

Pathways for formation of estrogen-like metabolites

Novel, estrogen-derived compounds, recently reported,[12–15] are based on the steroid core cyclopentanophenanthrene-4-ring, that is, similar to cholesterol, a precursor of bile acids, and steroid hormones including estradiol.[34] It is feasible that *S. haematobium* produces estradiol-related compounds, given that this parasitic flatworm may have evolved orthologous genes to those in mammals involved in estrogen metabolism.[35] Estradiol-like metabolites of schistosome origin may underlie the development of SCC of the bladder during UGS.[36]

Mammalian and related hormones, estrogen-like metabolites, and schistosome physiology

Whereas mammalian host hormones are required for the survival, development, and differentiation of schistosomes, the underlying molecular mechanisms remain unclear.[36,37] The Asian schistosome, *Schistosoma japonicum*, expresses receptors for steroid hormones displaying pathways for processing steroid hormones to anabolize derivatives.[38] The schistosome genome encodes putative enzymes that could convert progesterone and pregnenolone to estriol, estrone androsterone, and testosterone. Hence, schistosomes might exploit this pathway during their parasitic developmental stages.[38] A gene encoding an ortholog of 17β-hydroxysteroid dehydrogenase has been identified in the genome of *S. haematobium*. Orthologs of 17β dehydrogenase also occur in *S. mansoni* and *S. japonicum*, but given that the infection with these species of schistosomes has not been associated with cancer development, unlike the chronic infection with *S. haematobium*,[36] among other factors, it is not unreasonable to speculate that specific host tissues exposed to *S. haematobium* eggs, that is, urothelium, might be more susceptible to DNA damaged induced by these metabolites than host tissues exposed to *S. mansoni* and *S. japonicum eggs*, that is, liver and intestine.[35] Moreover, the genomes of these three schistosomes likely encode orthologous enzymes involved on steroid biosynthesis and steroid hormone biosynthesis pathways (Supplementary Figures S1 and S2). Characterization and functional analysis of these enzymes, summarized in Tables 1 and 2, employing functional genomic tools such as transgenesis, gene silencing, and CRISPR-Cas9-mediated genome editing might provide in the near future insights in their biological roles in the host-parasite relationship including in UGS-induced SCC.[39–41]

Role of P450 and reactive oxygen species (ROS) in the synthesis of metabolites

Steroid-related molecules may be metabolized into catechol-like compounds (intermediately producing ROS) that react with DNA via Michael addition forming DNA adducts and creating apurinic sites that could eventually lead to mutations further initiating a carcinogenesis process. Diverse cytochrome P450 enzymes play a key role both in the formation of estrogen and its subsequent oxidative metabolism as well in cholesterol catabolism to bile acids,[42] and also as a source of ROS.[43] The oxygenated metabolites of estrogens represent structures with newly generated hydroxyl and keto functions at specific sites in the steroid nucleus, which are analogous to other steroid categories that undergo oxidative metabolism, specifically androgens, vitamin D, and bile acids.[44] As shown in Figure 2, the conversion of catechol semiquinones to quinones, performed by these types of

Table 2. Orthologs of enzymes of the steroid hormone biosynthesis pathway orthologous enzymes encoded in the genomes of *Schistosoma haematobium* and *S. mansoni*.

Enzyme ID	Name	*S. haematobium*		*S. mansoni*	
		E-value	Acc. no.	E-value	Acc. no.
1.3.1.22	3-Oxo-5-alpha-steroid 4-dehydrogenase 1	3.50E−12	A 03363	–	–
1.3.1.3	3-Oxo-5-beta-steroid 4-dehydrogenase	–	–	1.30E−69	Smp 150700.1
1.1.1.146	Corticosteroid 11-beta-dehydrogenase isozyme 1	–	–	3.50E−14	Smp 186960.1
1.1.1.53	3Alpha(or 20beta)-hydroxysteroid dehydrogenase	8.40E−25	A 06678	5.50E−28	Smp 042680.1
1.1.1.62	17Beta-estradiol q7-dehydrogenase	1.00E−136	A 01343	2.20E−166	Smp 009430.1
1.14.14.1	Cytochrome P450, family 1, subfamily A, polypeptide 1	8.50E−15	A 00472	1.70E−12	Smp 156400.1
1.1.1.64	Testosterone 17beta-dehydrogenase (NADP+)	4.20E−46	A 05292	2.00E−46	Smp 168550.1

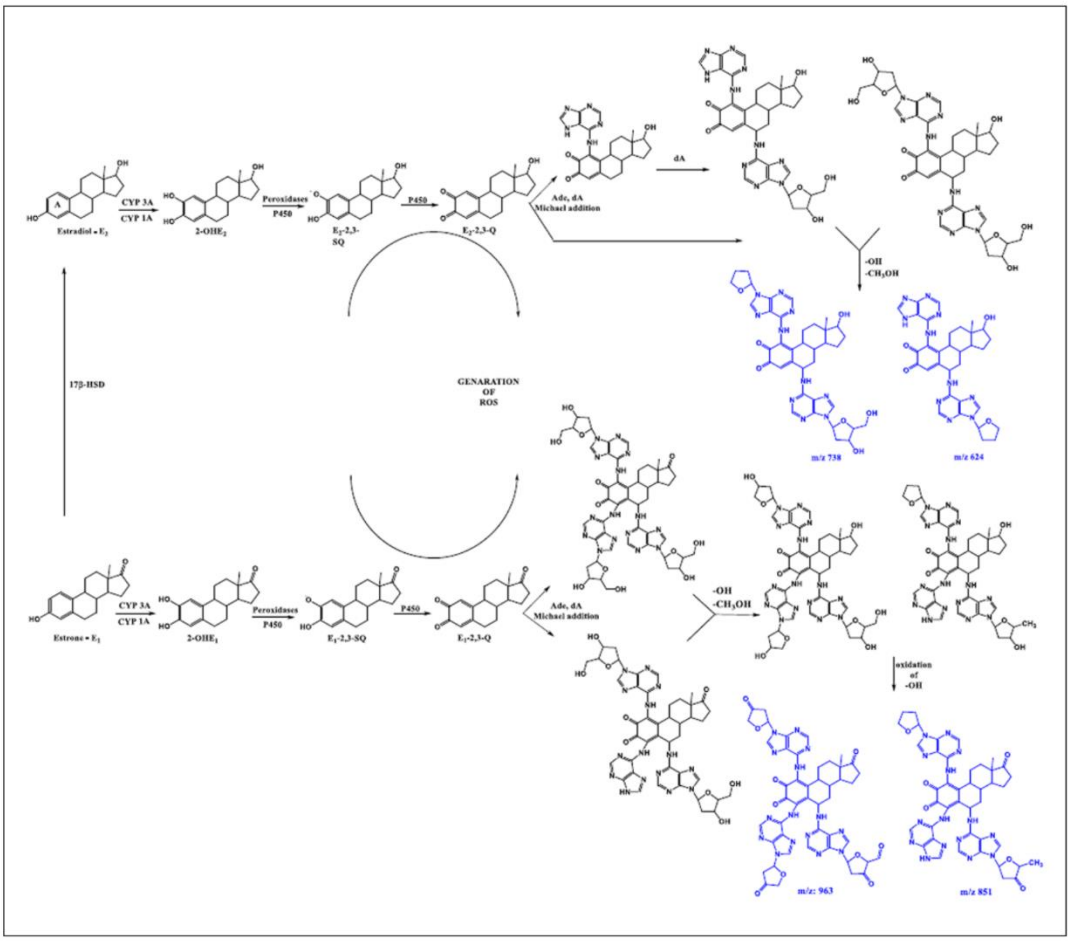


Figure 2. Hypothetical pathway for formation of molecules based on estrogen metabolism mediated by P450 enzymes in *Schistosoma haematobium* and in sera of persons with urogenital schistosomiasis. The initial step is oxidation of estrone (E_1) to estradiol (E_2) performed by 17β-HSD. E1 and E2 are metabolized to 2-hydroxy forms [2-OHE_1(E_2)], and then oxidized to catechol quinones [E_1(E_2)-2,3-Q]. P450 enzymes catalyze the conversion of catechol semiquinones [E_1(E_2)-2,3-Q] to quinones, which may release reactive oxygen species (ROS). Catechol quinones are reactive electrophiles capable of reaction with DNA bases, including adenine (Ade) and deoxyadenosine (dA) to form DNA adducts with structures compatible with m/z values established here (m/z 624, 738, 851, 963).

enzymes, can also enter into redox cycling and, thereby release ROS.[45] Accordingly, since *S. haematobium* exhibits P450 activity[28] suggesting that it is able to produce catechol compounds that lead to formation of ROS, this may contribute to oxidative stress during UGS. Increased oxidative stress associated with schistosomes has been described.[14] When ROS accumulate and/or when antioxidants defenses are saturated, the negative effects of oxidants ensue.[46] ROS induce the oxidation of DNA bases to yield 8-oxodG.[18] Thus, P450 through oxidative metabolism could lead to both oxidative and DNA adducts, with mutagenic potential.[31] The carcinogenic process in the bladders of the mice exposed to parasite antigens is independent of the effect of nitrosamines, findings indicating that schistosome antigens/metabolites induce inflammation.[47]

Estrogen-like metabolites and DNA adducts

There is a stepwise series in biosynthesis of estrogen from cholesterol. In brief, the biosynthesis commences with formation of pregnenolone catalyzed by CYP11A. This is succeeded by the formation of the androgens androstenedione and testosterone, catalyzed by CYP17, and is completed when CYP19 catalyzes aromatization of androstenedione to yield estrone (E_1) and estradiol (E_2).[48] Estradiol (E_2) is mainly metabolized by (i) oxidation of hydroxyl function at the C-17 position to yield estrone (E_1). 17β-Hydroxysteroid dehydrogenase (17β-HSD), a key enzyme in the metabolism of estrogen,[49] catalyzes this conversion. Estrogen is also metabolized by hydroxylation via cytochrome P450 enzymes, preferentially on C2, C4, and C16 positions. When hydroxylation takes place on the steroid aromatic ring A, catechol estrogens are produced.[50–52] CYP1A1, CYP1A2, and CYP3A4 participate in this process; they exhibit catalytic activity dominantly for the 2-hydroxylation in estrogen[38] to liberate 2-hydroxyestrone/estradiol (2-$OHE_1(E_2)$). Thereafter, further oxidation to semiquinones (E_1E_2-2,3-SQ) proceeds, and to quinones (E_1E_2-2,3-Q) catalyzed by P450 enzymes. ROS also are liberated in this conversion. As electrophiles, catechol quinones form covalent adducts with DNA bases, such as adenine (Ade), and deoxyadenosine (dA), via Michael addition,[29,31,52,53] giving rise to structures compatible with those with m/z values 624, 738, 851, and 963 that have been identified in the peripheral blood during UGS (Figure 2).[13]

Cholesterol is converted into bile acids through discrete enzymatic steps that include initiation of synthesis by 7α-hydroxylation of sterol precursors, ring structure modification, oxidation and shortening of the side chain, and conjugation of bile acid with amino acid.[54,55] Microsomal cytochrome P450 enzyme cholesterol 7α-hydroxylase (CYP7A1) catalyzes the hydroxylation to yield 7α-hydroxycholesterol.[54] The product released from this hydroxylation is converted into 3-oxo Δ^4 forms by microsomal 3β-hydroxy-Δ^5-C27-steroidoxidoreductase (HSD3B7) and forms 4-cholesten-7α-ol-3-one.[54] The enzyme CYP8B1, a sterol 12α-hydroxylase, promotes the 12-hydroxylation leading to formation of 4-cholesten-7α-12α-diol-one.[54,55] Thereafter, AKR (aldo-keto reductase) family enzymes modify the aromatic ring. AKR1D1 and AKR1C4 act in concert, where AKR1D1 catalyzes 5β-reduction of the bile precursor from 4-cholesten-7α-12α-diol-3-one to 5β-cholesten-7α-12α-diol-3-one; AKR1C4 catalyzes the further reduction of ketogroups[56] forming 5β-cholesten-3α-7α-12α-triol. The next step is the side chain hydroxylation leading to form 5β-cholesten-3α-7α-12α-27-triol performed by CYP27A1. CYP27A1 introduces a hydroxyl group at C27 position and oxidizes this group to an aldehyde and subsequently to a carboxylic acid[55,57,58] leading to 3α-7α-12α-trihydroxy-5β-cholestanoic acid. Bile acid CoA ligase, CoA racemase, AcylCoA oxidase, D-bifunctional enzyme, and peroxisomal thiolase 2 modify the product to 3α-7α-12α-trihydroxy-5β-cholan-24-one-CoA.[55,56] The terminal step of primary bile acids formation is the conjugation with an amino acid, usually glycine or taurine, and occurs in amide linkage on C24. Bile acid coenzyme A: amino-*N*-acyltransferase catalyzes the reaction. The latter is a notably efficient enzyme, given that >98% of bile acids excreted from liver are amidated.[59] The substrates are a bile acid coenzyme A thioesther (3α-7α-12α-trihydroxy-5β-cholan-24-one-CoA) and glycine and taurine.[59] The ultimate step liberates glycocholic or taurocholic acid.

As obligate parasites, the blood flukes likely evolved the capacity to exploit host hormones for their own needs, deploying the enzyme P450 to release $E_1(E_2)$-2,3-Q and related metabolites including catechols and other electrophiles (Figure 2). These metabolites are generally inactivated by conjugation reactions including methylation, glucuronidation, and/or sulfation. The common pathway of conjugation in extrahepatic tissues occurs by *O*-methylation catalyzed by the ubiquitous catechol-*O*-methyltransferase (COMT).[53] If conjugation of catechol estrogens via methylation becomes insufficient, competitive catalytic oxidation of catechol estrogen to catechol estrogen quinones can proceed.[53] Alterations in the activity of phase I and phase II drug-metabolizing enzymes in the urothelium of the bladder during UGS might arise, interfering with the detoxification of xenobiotics and other homeostatic processes. Indeed, diminished glutathione-*S*-transferase activity occurs during UGS.[46] If homeostatic detoxification of catechols was impeded during UGS, these metabolites might accumulate in the urothelium and elsewhere.

Pioneering reports indicated that schistosomes could not synthesize sterols *de novo*.[60] However, this is controversial given that lysates of schistosomes convert steroids to related metabolites.[42,61] Following infection of the skin penetration, the schistosomules migrate in the circulation to the lungs, after which the parasites enter the

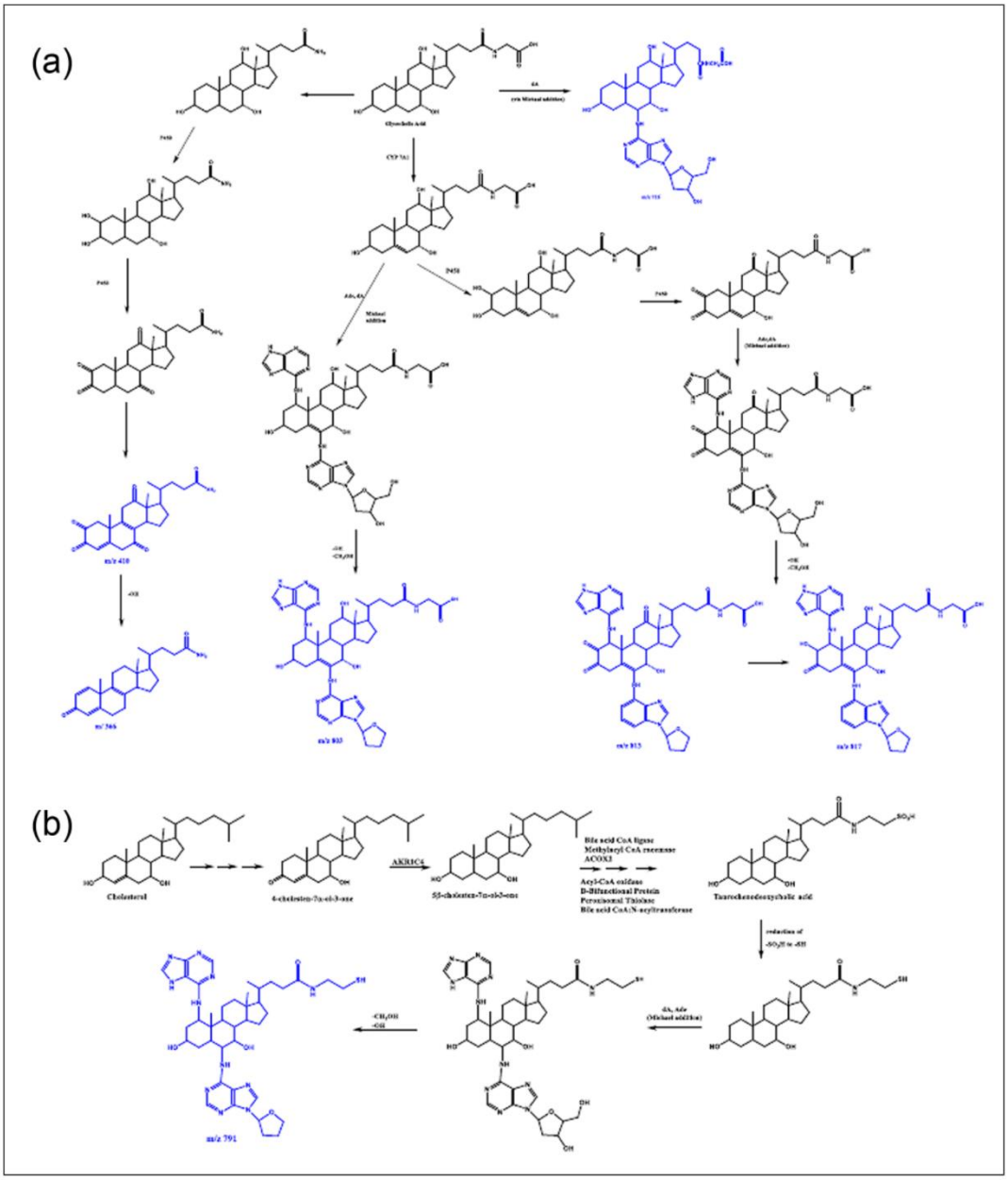


Figure 3. Possible pathway formation for estradiol-like metabolites reported in eggs and adult stages of *Schistosoma haematobium* and in urine of patients with urogenital schistosomiasis (UGS), derived from glycocholic acid (a) and taurochenodeoxycholic acid (b). The molecules with m/z 366, 410, 791 were observed in urine during UGS with or without bladder cancer. Metabolites with m/z 813 and 817 were observed in schistosomes. The compound with m/z 803 was observed in urine during UGS and also within the parasite itself.

hepatic portal circulation or the vessels of the pelvic organs depending on the species; *S. haematobium* adult worms dwell in the venous plexus of the bladder and genital tract, whereas the other major human species dwell in the mesenteric veins.[62]

Catechol-like metabolites produced either by non enzymatic process (autoxidation by oxidative free radicals) or enzymatic catalysis[42] might react with DNA bases to yield the molecules we have proposed (Figure 3). Based on recent findings,[9,10,12,16] glycocholic acid may (1) lose the acidic moiety (-CH_2COOH) and undergo hydroxylation then oxidation to produce the catechol-like compound m/z 410; (2) lose oxo groups, and introduce a double bond on aromatic ring A to form the compound with m/z 366; (3) forms DNA adducts by reacting with DNA bases via Michael addition, m/z 716; (4) or after aromatic ring

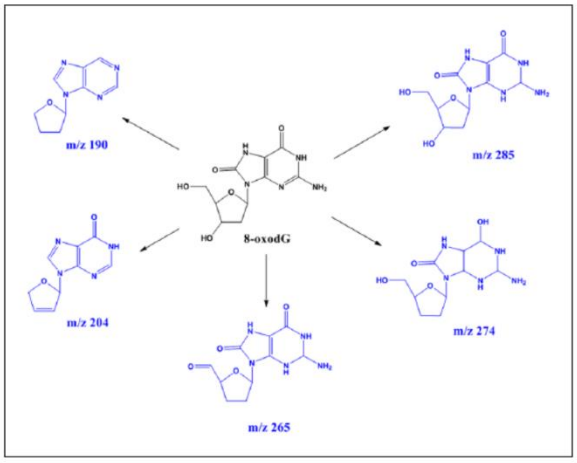


Figure 4. DNA damage possibly related to estrogen-like metabolites from parasite. Compounds in urine of persons infected with the blood fluke *S. haematobium* with or without infection-induced bladder cancer. The compounds derive directly from 8-oxodG, a product of DNA oxidation. Formation of these molecules may be a consequence of interaction with catechol compounds from parasite.

modifications and further reaction with DNA bases to form m/z 803; or (5) undergo through a hydroxylation at C2 position followed through an oxidation and then react with DNA forming m/z 813. This compound could undergo through a reduction of some hydroxyls group forming the compound with m/z 817 (Figure 3(a)). Other compound proposed (Figure 3(b)) differs in some ways from the above. Instead of a glycocholic acid, the precursor is a taurochenodeoxycholic acid. The synthesis is similar to that for glycocholic acid, but this compound does not proceed through hydroxylation of CYP8B1. Rather than forming 5β-cholesten-7α-12α-diol-3-one, 5β-cholesten-7α-ol-3-one is liberated (Figure 3(b)). This product undergoes the same reactions of 5β-cholesten-7α-12α-diol-3-one, but the reaction conjugates the amino acid taurine instead of glycine. The next step is the reduction of sulfonic acid moiety ($-SO_3H$) of taurochenodeoxycholic acid to a thiol group (-SH). Again, this product could eventually react with DNA bases leading to the compound with m/z 791. The estrogen-like DNA adducts with m/z 716, 803, 813, 817 have been described in *S. haematobium*;[14] the remaining compounds highlighted here, m/z 366, 410, 791, have been seen in urine during UGS including cases with or without bladder cancer. In addition, the m/z 803 metabolite was identified in both eggs and other developmental stages of the schistosome, and in urine of patients with UGS.[14,15]

Consequences of estrogen-like metabolites

The formation of DNA adducts induces continuous DNA oxidation, leading to mutations and/or gene expression dysregulation of oncogenes and tumor suppressors, such as the overexpression of *p53* observed during UGS.[63] Consequently, these metabolites of estrogens derived from the parasite might behave as chemical carcinogens.[47] In addition, there may be "classical" hormone-like effects of these metabolites on the endocrine and immune system of the host; the ER activity was suppressed in urothelial cells cultured in vitro and in the bladders of mice exposed to *S. haematobium* antigens.[64]

The levels of oxidized DNA bases reflect a balance between the amount of oxidative DNA damage and DNA repair. It seems reasonable to assume that a suboptimal repair of 8-oxodG would be mutagenic, and may be implicated in the initiation and/or promotion of inflammation-mediated carcinogenesis.[47,65,66] During UGS, in the face of continuous shedding of parasite eggs into the wall of the bladder and egress of the eggs with the urine, sustained production of catechols is expected, with continuous generation of ROS that oxidize DNA bases of urothelial cell chromosomes. Cavalieri and coworkers postulate that the presence of high levels of oxidized DNA suggests a carcinogenic mechanism induced by catechol estrogens.[52] Among the compounds related to DNA adducts, we observed metabolites with m/z 285, 274, 265, 204, 190 (Figure 4), derived directly of 8-oxodG in patients with UGS and UGS-induced bladder cancer.[15] Higher levels of metabolites derived from 8-oxodG were identified in persons with UGS free of cancer than in patients with already advanced UGS-induced bladder cancer. Hence, we speculate that DNA oxidation may be more pronounced during incipient cancer than where the UGS-induced bladder cancer has already manifested.[15] In addition, 8-nitroguanine forms via inducible expression of nitric oxide synthase in Oct3/4-positive stem cells in UGS-associated bladder cancer tissue,[67] and DNA nitrative and oxidative mutations characterized by 8-nitroguanine and 8-hydroxy-2′-deoxyguanosine (8-oxodG), have been implicated in the promotion of inflammation-mediated carcinogenesis by infection with *S. haematobium*.[68] Moreover, other studies have shown that UGS is likely to cause bladder cancer by this mechanism, and noted a correlation between UGS and increase of levels of oxidative stress accompanied by continuous DNA damage and repair in urothelial carcinomas.[20,69] These metabolites might damage DNA, for example, by formation of DNA adducts (m/z 624, 716, 738, 803, 817, 851, and 963) and contribute to intermediate production of ROS during formation of catechol compounds (Figures 2 and 3). Furthermore, these phenomena may explain formation of 8-oxodG derivatives (as illustrated in Figure 4), metabolites known from urine during UGS.[15]

Overexpression of p53 *during UGS*

The *p53* is a tumor suppressor protein involved in diverse pathways including regulation of cell cycle, suppression of cell proliferation, cellular response to DNA damage,

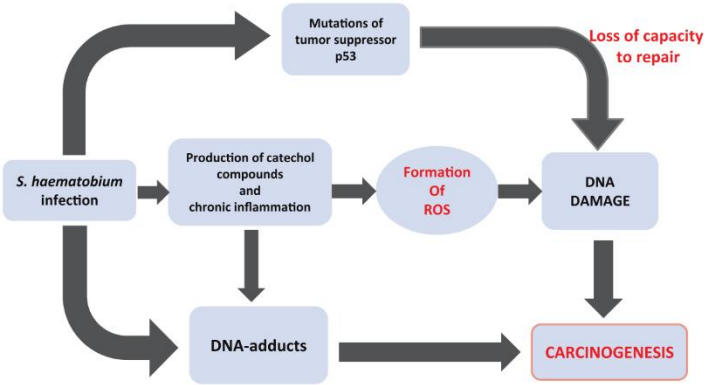
initiation of DNA repair and replication, induction of apoptosis, and promotion of differentiation.[27,28,66,70] Dysregulation of the expression of *p53* gene may accelerate the tumorigenesis and alter the response of cellular agents that damage DNA.[2] Together, the genes encoding these proteins are referred to as tumor suppressor genes. Inactivation or alteration in the expression of these genes together with activation of oncogenes leads to malignant transformation.[71] It has been shown that the formation of DNA adducts may be related to a high expression of *p53* during schistosomiasis.[28,72,73] Given that (i) *S. haematobium* produces compounds that react with DNA and damage chromosomal DNA, and (ii) *p53* mutations have been associated with schistosomiasis,[28] chronic infection with *S. haematobium* may stimulate the expression of *p53* during UGS and UGS-induced bladder cancer. In addition, *p53* is overexpressed during UGS, although the molecular mechanisms remain unclear,[20,23,29,69,74] and *p53* protein has been detected by immunolocalization in both aggressive urothelial and squamous cell bladder carcinomas. Notably, *p53* is mutated and not functional in benign/premalignant lesions, predominantly in those showing cellular alterations, including urothelial hyperplasia, epidermoid metaplasia, and/or dysplasia.[20] This conforms to findings in urine using liquid chromatography–mass spectrometry LC-MS/MS during UGS, where DNA oxidation processes appear more pronounced in cases with UGS but without cancer.[15] The eventual association between the dysregulation of *p53* in urothelium and infection with *S. haematobium* reinforces the notion that UGS profoundly alters in cellular process in the urothelium, ultimately giving rise to bladder cancer after a continuous accumulation of mutations and selection of aggressive clones of transformed cells. By employing a model of chemical-induced carcinogenesis of the bladder in genetically modified mice, that is, using *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), it has been recently demonstrated the cellular origin of the bladder neoplasia.[75] In this model, invasive carcinoma is initiated from basal urothelial stem cells that started to accumulate mutations that eventually offer positive selection of aggressive clones of cells. The probability of this phenomenon ultimately depends on the mutagenesis pressure on the target tissue that in the context of UGS where the described estrogen-like metabolites are produced may be higher.[15]

Future perspectives

Carcinogenic transformation of the bladder during UGS may proceed along a pathway involving the formation of catechol and DNA adducts, intermediary formation of ROS, *p53* mutations that inhibit the repair of DNA and, in turn, initiate carcinogenesis (Box 1). Whereas carcinogenesis may be induced by other factors,[76,77] this is a plausible route to UGS-induced SCC. Pathways for formation of these carcinogenic metabolites with parasite origin are not yet fully understood. Investigations in this field are required to determine specific enzymatic functions of pathogens and whether these correlate with the ability of various Schistosoma species to produce bladder cancer. The findings hold the promise for new interventions, including prognostic biomarkers and indeed a vaccine against this NTD-induced cancer.

Box 1. Outstanding questions.

- Schistosomiasis is considered the most important of the human helminth diseases in terms of morbidity and mortality. Three major species of schistosomes contribute to the affliction-*Schistosoma haematobium, S. mansoni*, and *S. japonicum*. However, why infection with *S. haematobium* is carcinogenic whereas infection with other schistosomes is not remains to be resolved.
- What are the physiological roles of schistosome estradiol-like metabolites for the parasite, in view of their (additional?) capacity as carcinogens in bladder cancer during UGS; unfortunate and unwelcome bystander effect?
- Does the genome of the blood fluke encode the necessary pathways to synthesize endogenous estradiol *de novo* and/or does the blood fluke catabolize and modify hormones of human origin for its purposes?



Pathways to initiation of carcinogenesis provoked by urogenital schistosomiasis, infection with Schistosoma haematobium

Declaration of conflicting interests

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Appendix 2

Praziquantel for schistosomiasis: single-drug metabolism revisited, mode of action, and resistance

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MINIREVIEW

Praziquantel for Schistosomiasis: Single-Drug Metabolism Revisited, Mode of Action, and Resistance

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ABSTRACT Schistosomiasis, a major neglected tropical disease, affects more than 250 million people worldwide. Treatment of schistosomiasis has relied on the anthelmintic drug praziquantel (PZQ) for more than a generation. PZQ is the drug of choice for the treatment of schistosomiasis; it is effective against all major forms of schistosomiasis, although it is less active against juvenile than mature parasites. A pyrazino-isoquinoline derivative, PZQ is not considered to be toxic and generally causes few or transient, mild side effects. Increasingly, mass drug administration targeting populations in sub-Saharan Africa where schistosomiasis is endemic has led to the appearance of reduced efficacy of PZQ, which portends the selection of drug-resistant forms of these pathogens. The synthesis of improved derivatives of PZQ is attracting attention, e.g., in the (i) synthesis of drug analogues, (ii) rational design of pharmacophores, and (iii) discovery of new compounds from large-scale screening programs. This article reviews reports from the 1970s to the present on the metabolism and mechanism of action of PZQ and its derivatives against schistosomes.

KEYWORDS cytochromes P450, enantiomers, metabolism, praziquantel, schistosomiasis

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Schistosomiasis, a major neglected tropical disease, is considered the most important helminthic disease of humanity in terms of morbidity and mortality rates. More than 200 million people are infected worldwide, and 600 million are at risk of infection (1, 2). Control strategies have been employed to block transmission and reduce the disease burden, including mass and targeted chemotherapy, improvements to sanitation, modification of the environment, and the use of molluscicides (3, 4). However, schistosomiasis remains a major public health problem, especially in rural regions of sub-Saharan Africa (2). The infection is caused by three main species of blood flukes, *Schistosoma haematobium* in Africa and the Middle East, *S. mansoni* in Africa and South America, and *S. japonicum* in China and the Philippines, and two less common ones, *S. intercalatum* in Africa and *S. mekongi* in Southeast Asia (5). Moreover, recent outbreaks reveal the reemergence of urogenital schistosomiasis in southern Europe (6). Additionally, infection with *S. haematobium* is classified as a group I biological carcinogen by the International Agency for Research in Cancer of the World Health Organization (WHO) (7). Table 1 summarizes the species that commonly infect humans, the geographical ranges of endemicity, and the major disease symptoms (5, 7, 8).

Male and female schistosomes dwell in copula within the mesenteric veins (*S. mansoni*, *S. japonicum*) or the venous plexus (*S. haematobium*) of the human host,

TABLE 1 *Schistosoma* species, regions of prevalence, and major signs and symptoms of schistosomiasis

Species	Regions of prevalence	Pathology, symptoms, signs
S. mansoni	Africa, Middle East, Caribbean, South America	Liver/periportal fibrosis, hepatomegaly, intestinal fibrosis, diarrhea
S. japonicum	China, Southeast Asia (Philippines, Indonesia)	Blood in stool, portal hypertension, hepatomegaly, intestinal fibrosis, diarrhea, blood in stool, CNS[a] complications
S. mekongi	Cambodia, Lao People's Democratic Republic	Same as for *S. japonicum*
S. hematobium	Africa, Middle East, southern Europe (Corsica, France)	Urogenital tract fibrosis, female genital schistosomiasis, bladder cancer, renal failure, infertility

[a]CNS, central nervous system.

laying hundreds to thousands of fertilized eggs per day, depending on the species. The eggs traverse the intestinal wall (e.g., *S. mansoni*) or the bladder wall (*S. haematobium*) and exit the host to the external environment in feces or urine, respectively. However, many eggs are retained in host tissues, where they induce inflammation, granuloma, and fibrosis. In the external environment, the eggs hatch when they reach freshwater, releasing a free-living larva, the miracidium, that is ciliated and seeks to infect the obligate intermediate host, a snail. Within the snail, the parasite undergoes cycles of asexual reproduction through mother and daughter sporocyst stages, eventually shedding thousands of cercariae into the water. The cycles of asexual reproduction of the parasite within the snail require from 4 to 6 weeks before cercariae are released. The cercaria is the infectious developmental stage for humans and other mammals. After penetrating the skin, the cercariae shed the tail and the juvenile larvae, termed schistosomula, migrate within the circulatory system, reaching the lungs, the liver, and finally the portal venous system or the venous system that drains the pelvic organs, depending on the species, where the parasite fully matures. Adult *S. mansoni* worms migrate to the superior mesenteric veins, *S. japonicum* worms migrate to the inferior mesenteric and superior hemorrhoidal veins, and *S. haematobium* worms migrate to the vesical plexus and veins draining the ureters, bladder, and other pelvic organs. Male and female schistosomes mate, produce eggs, and thus complete the developmental cycle (Fig. 1) (6).

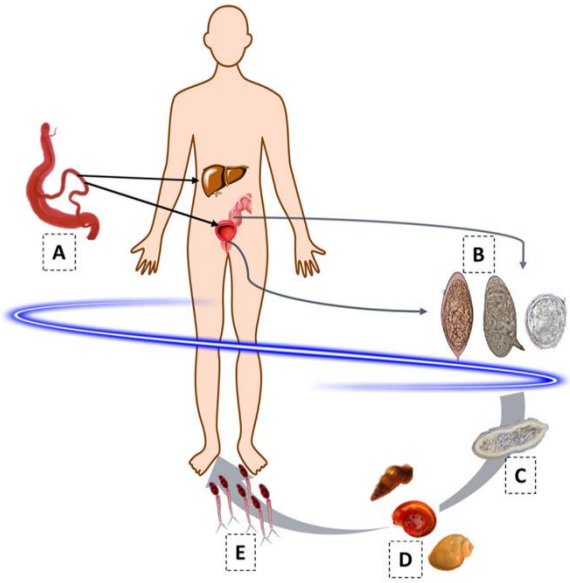

FIG 1 The developmental cycle of *S. mansoni*, *S. haematobium*, and *S. japonicum*. Stages: A, paired adult worms (larger male enfolding slender female); B, eggs (left to right, *S. haematobium*, *S. mansoni*, and *S. japonicum*); C, ciliated miracidium; D, intermediate host snails (left to right, genera *Oncomelania*, *Biomphalaria*, and *Bulinus*); E, cercariae (infective stage).

TABLE 2 Clinical phases of schistosomiasis and its associated symptoms[a]

Clinical phase	Symptoms
Immediate	Acute, pruritic, maculopapular eruption at site of cercarial skin penetration within 1 day following exposure
Acute	Systematic hypersensitivity reaction against migrating schistosomula, fever, fatigue, myalgia, malaise, nonproductive cough, eosinophilia, patchy infiltrates, weight loss, dyspnea, diarrhea, diffuse abdominal pain, toxemia, hepatosplenomegaly, widespread rash
Chronic	Affects gastrointestinal and urogenital tracts, leading to hepatosplenic and pelvic organ diseases, portal and pulmonary hypertension, abdominal ascites, upper gastrointestinal varices and hemorrhage, female genital schistosomiasis, infertility, increased risk of HIV-1 transmission, and squamous cell carcinoma of the bladder

[a]See references 7 and 9 to 13.

The infection clinically progresses from an immediate phase to acute and chronic stages (7, 9–13). The initial phase is typically characterized by an acute, pruritic, maculopapular eruption at the site of cercarial skin penetration within the first 24 h after exposure. This may last several days, may occur even with zoonotic schistosome species that do not usually mature in humans, and is also known as cercarial dermatitis or swimmer's itch. Acute schistosomiasis (Katayama fever) is a systemic hypersensitivity reaction to the migrating schistosomula that occurs a few weeks to months after a primary infection. The disease starts suddenly with fever, fatigue, myalgia, malaise, nonproductive cough, eosinophilia, and patchy infiltrates on chest radiography. Abdominal symptoms develop later, following the migration and residence of the mature worms in the blood vessels of the intestines and bladder. Most persons recover spontaneously from the acute stage after 2 to 10 weeks, but some develop a persistent and more serious disease with weight loss, dyspnea, diarrhea, diffuse abdominal pain, toxemia, hepatosplenomegaly, and a widespread rash (7, 9, 10). During chronic or advanced schistosomiasis, which can persist for decades in the absence of treatment, the gastrointestinal and urogenital tracts are affected, leading to hepatosplenic and pelvic organ diseases and other complications, including portal and pulmonary hypertension, abdominal ascites, upper gastrointestinal varices and hemorrhage, infertility, and increased risk of HIV-1 transmission (Table 2) (10–13).

The paucity of information on new derivatives of praziquantel (PZQ1) is curious, especially since not only is schistosomiasis one of the major neglected tropical diseases but infection with *S. haematobium* is a biological carcinogen (14). Neglect of the latter undoubtedly relates to the lack of reliable rodent models of urogenital schistosomiasis. Nonetheless, the design of novel, rational compounds with potential antischistosomal activity is hindered by the absence of the definitive mode of the antischistosomal action of PZQ1. Although investigation of novel PZQ1 derivatives apparently continues, there is not a wealth of information available on the mode of drug action. Here, we review recent developments on derivatives of PZQ1, including activity and metabolites, as well as modes of action and drug resistance. We believe that review of this information will be beneficial for the identification of novel antischistosomal drugs and new drug targets.

A SINGLE DRUG FOR TREATMENT AND CONTROL OF SCHISTOSOMIASIS

The pyrazino-isoquinoline derivative PZQ1 (Fig. 2) was developed by Bayer in the 1970s and shown to be active against parasitic flatworms, including schistosomes. Remarkably, treatment and control of schistosomiasis have relied only on this drug for over 40 years (15–17). In animal tests, PZQ1 showed minimal toxicity (18) and no genotoxic risks (19) were detected in assays for mutagenicity (20). The few observations that suggested accumulation of potentially mutagenic metabolites may have been abnormalities among otherwise overwhelming evidence indicating that PZQ1 is a safe drug (21). Generally, PZQ1 induces only mild and transient side effects, if any. The

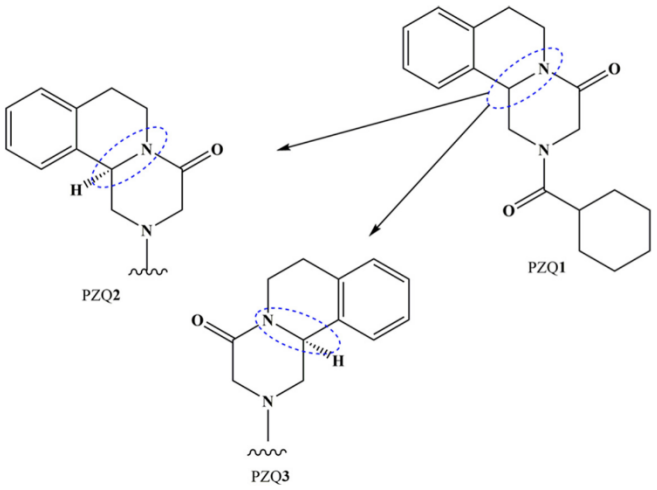

FIG 2 Enantiomers of PZQ1 and biologically active (*R*-PZQ, PZQ2) and inactive (*S*-PZQ, PZQ3) isomers.

frequency and intensity of these effects are correlated with the intensity of infection, and the most severe side effects of bloody diarrhea or edematous urticaria observed in areas with high intensities of infection may be due to the release of antigens and other metabolites by dying worms (22, 23).

During the past few years, the renewed acknowledgment of the burden imposed by schistosomiasis has led to the implementation of mass drug administration (MDA) programs for the control and possible elimination of the this major human helminthiasis, yet the WHO recently reported that less than one-third of individuals who required "preventive chemotherapies" received treatment (24). PZQ1 has been widely used since 2006 through "preventive chemotherapy" programs distributing the drug to school age children or at-risk populations, depending on prevalence rates. In 2010, 34 million people received PZQ1, mostly in sub-Saharan Africa (16). It has been estimated that by 2018, as many as 235 million people will have been treated with PZQ1, a projected use of 645 million tablets of PZQ1 (25). Also, PZQ1 is effective in the treatment of hypertension due to chronic schistosomiasis (10). This continues to be a key drug in the treatment of schistosomiasis and, indeed, most other fluke and cestode infections (17).

According to the Biopharmaceutics Classification System and the Biopharmaceutics Drug Disposition Classification System, PZQ1 is a class II drug that displays a high ability to permeate tissues and low solubility (0.4 mg/ml) and proceeds through extensive metabolism (discussed below) (26, 27) via hydroxylation of the absorbed drug to inactive metabolites, such that only minimal concentrations contact the parasites within the blood system. Currently, PZQ1 is distributed as a racemate that includes equivalent proportions of the biologically active *R*-PZQ (PZQ2, Fig. 2) and inactive *S*-PZQ (PZQ3, Fig. 2) (28) enantiomers, the consequence of which is that half the PZQ1 dose is pharmacologically inactive. This requires the use of a 600-mg tablet to provide a final dose of 40 mg/kg. Moreover, PZQ3 probably contributes to the unpleasant taste of PZQ1. These disadvantages contribute to inefficient treatment of school age children, since children frequently avoid swallowing the medicine because of its less-than-pleasant taste (28). Meyer et al. (29) investigated the bitterness value of enantiomers in regard to additional incentives for low-cost production of pure active PZQ1 (29). Indeed, the pure enantiomer of PZQ2 can probably be synthesized economically (30, 31). Among these variants, however, PZQ1 presents other disadvantages, such as decreased or complete absence of activity against juvenile schistosomes (32, 33). Accordingly, a complete cure is not reliably achieved with a single dose of PZQ1, particularly given that reinfection is routine (8, 34).

Despite decades of extensive use, much remains unknown about PZQ1, in particular, its exact mode of action, its *in vivo* metabolism, and its molecular target(s). Herein, these aspects are reviewed along with prospective derivatives of PZQ1.

PZQ PHARMACOKINETICS

Although PZQ1 has been employed for decades, few pharmacokinetic studies have been performed with humans (26), although a study carried out with healthy volunteers demonstrated that absorption of PZQ1 is relatively fast (time to maximum concentration of drug in serum [T_{max}], 2.0 to 2.6 h) and nearly complete (>80%), which demonstrates an extensive first-pass effect (35). The systemic bioavailability of PZQ1 is low and varies considerably among individuals. After the administration of 40 mg/kg to a healthy adult, the half-life ($t_{1/2}$) was reported to range from 2.2 to 8.9 h and the area under the curve (AUC) was reported to range from 2,100 to 5,400 ng h/ml. Oral drugs display higher pharmacokinetic variability than drugs administered by the parenteral route, which is explained by the blood flow at the absorption site, the absorptive surface area, the transit time, and the gastric pH (36). These factors are also influenced by concurrent food intake; the bioavailability of PZQ1 increases with concomitant food administration. Following the administration of 1,800 mg (~25 mg/kg for a 70-kg body weight) to healthy adults, the AUC from 0 to 8 h was 2.7-fold higher with a fatty diet and ~4 times as high with a high-carbohydrate diet (37). The effect of food on bioavailability may also be due to changes in hepatic flow, altered cytochrome P450 (CYP) expression in response to the diet, or changes in the first-pass metabolism of PZQ1 (38, 39). The bioavailability of PZQ1 has also been analyzed during schistosomiasis. Comparing the bioavailability of PZQ1 in healthy volunteers and infected people after the administration of 40 mg/kg, the C_{max} (the maximum or peak concentration) and AUC were 1.7- and 4.2-fold higher in patients, the T_{max} was 0.6 times shorter, and the $t_{1/2}$ was 5.2 times longer (40). PZQ1 is mainly concentrated in the liver and kidneys. Concentrations higher than those in plasma occur in the lungs, pancreas, adrenal glands, pituitary gland, and salivary glands (41). However, the volume of distribution is not known (41). In addition, PZQ1 binds to proteins (~80% exclusively to albumin). Hence, nutritional status and other factors, including chronic inflammation, influence the levels of the free drug (35, 42).

INSIGHTS INTO METABOLISM OF PZQ

As noted, PZQ1 is mainly metabolized to PZQ2 and PZQ3, which in turn breaks down into various mono- or dihidroxy metabolites and *S-trans*- and *S-cis*-4-OH-PZQ, while PZQ2 is metabolized to *R-trans*-4-OH-PZQ or *R-cis*-4-OH-PZQ (Fig. 3) (43–45). Since higher drug concentrations in plasma and slightly longer half-lives are achieved with metabolites than with PZQ1, the metabolites likely contribute to the drug's antischistosomal activity (46). In fact, *in vitro* studies using PZQ2 and PZQ3 and its major metabolites against *S. mansoni* developmental stages (newly transformed schistosomula and adult worms) demonstrated that PZQ2 and its metabolites exhibit 100- and 1,000-fold higher activities than their *S* counterparts. These findings confirm that PZQ2 is the main effector, whereas PZQ3 and its metabolites do not contribute significantly to the drug's antischistosomal activity (15). Nonetheless, metabolites of PZQ1 are less active than the parent drug (47). Although the enzymes that metabolize PZQ1 are not fully known, PZQ1 is primarily metabolized by CYP 3A and to a lesser extent by CYP 2D6 (35). Several studies have been performed to clarify the metabolic profile of PZQ1, as well as the enzymes involved and the identities of the phase II metabolites (48–53).

Development of new PZQ derivatives might be a good strategy to circumvent the major drawbacks of current PZQ1 therapy. Substantial investigation has been directed to the design of different types of PZQ derivatives. Through the years, several PZQ derivatives have been developed and assessed via *in vitro* and *in vivo* studies mainly against *S. mansoni* and *S. japonicum*. Design of urea and amide derivatives (Fig. 4) led to a moderate reduction of worm motility *in vitro*, but generally, this activity was not observed *in vivo*. However, one derivative of these series, PZQ7, stood out in regard to

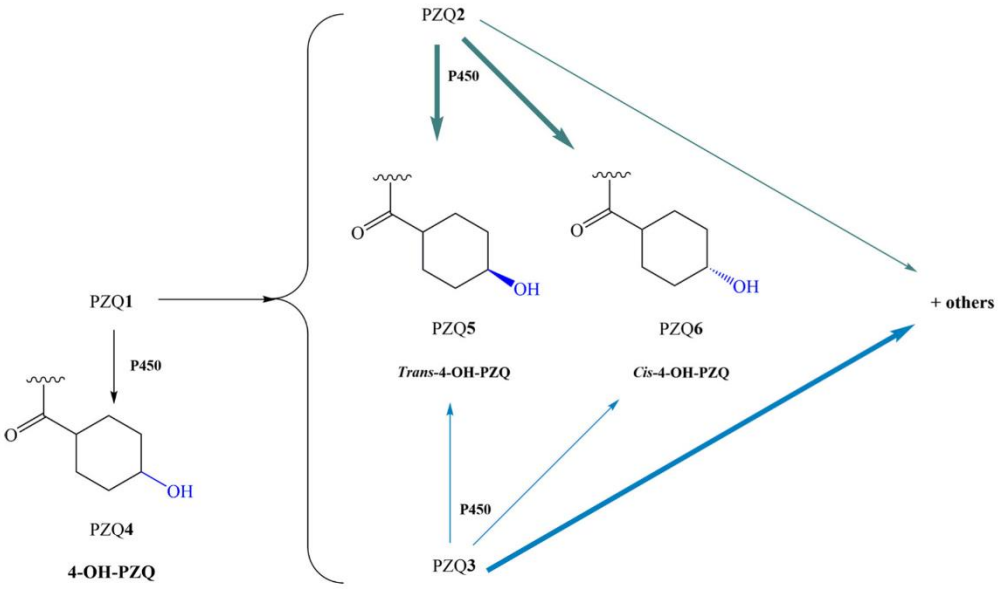

FIG 3 PZQ1 is metabolized by CYP 450, resulting in PZQ4 as the main product and other minor enantiomers such as PZQ5 and PZQ6. In addition, enantiomers of PZQ1 also undergo metabolism. Bold green and blue arrows indicate major metabolites of PZQ enantiomers PZQ2 and PZQ3, respectively. PZQ2 is mainly metabolized into *trans*- and *cis*-4-OH-PZQ (PZQ6), whereas PZQ3 is mainly metabolized into other mono- or dihydroxylated forms of PZQ1 and, to a less extent, into *trans*- and *cis*-4-OH-PZQ (PZQ5, PZQ6). P450 enzymes perform these transformations.

significant activity *in vivo*, which may derive from its *in vivo* reduction to a *trans*-cyclohexanol metabolite, PZQ4 (54). With this structure in mind, chiral PZQ derivatives (PZQ10 and PZQ11) were developed, as well as chiral PZQ7. Racemic PZQ10 and PZQ11 had modest activity against *S. japonicum in vitro*, while their enantiomers (*S* and *R*) failed to display any activity, with the exception of racemic PZQ7 and its enantiomers. Overall, all chiral PZQ derivatives display modest activity *in vivo* compared to that of PZQ1. Intriguingly, the size of the ring with a carbonyl group in these derivatives had an appreciable impact on *R* isomers, increasing their activity, but had almost no effect on *S* isomers (55). Using organometallic moieties such as ferrocenyl (already present in anticancer, antibacterial, and antimalarial drugs [56–58]) Fc-PZQ derivatives (types A and B, Fig. 4) displayed anthelmintic activity in the micromolar range but were considerably less active than PZQ1 (59). Upon alteration of the organometallic moiety to $Cr(CO)_3$ (CrPZQ16, CrPZQ17, Fig. 4), the derivatives exhibited marked activity against *S. mansoni in vitro*; however, they exerted low activity *in vivo* (60). This fact might be related to the metabolite liability of these derivatives, resulting in less-active metabolites (Fig. 5), and due to protein binding or distribution (61). Following a different design strategy, a combination of artesunate (AS) and PZQ1 led to new derivatives that incorporate these two moieties, DW-3-15. Because of the complementary effect of these two drugs (AS is more effective against schistosomula and juvenile worms, whereas PZQ is effective against adult worms), it was expected that this derivative would demonstrate potential broad-spectrum antischistosomal activity (62, 63). In fact, biological evaluation of DW-3-15 (Fig. 4) proved that the complementary functions of AS and PZQ1 were more effective than PZQ1 alone. Therefore, this might encourage rational drug design by combining pharmacophore moieties of discrete bioactive compounds with dual modes of action (62, 63). In comparison to DW-3-15, it was expected that endoperoxide-PZQ derivatives would have increased bioavailability (since their molecular weight was less than that of DW-3-15) and thereby improved antischistosomal activity. Although these derivatives presented good efficacy against adult *S. mansoni* worms *in vitro*, their activity was lower than that of DW-3-15.

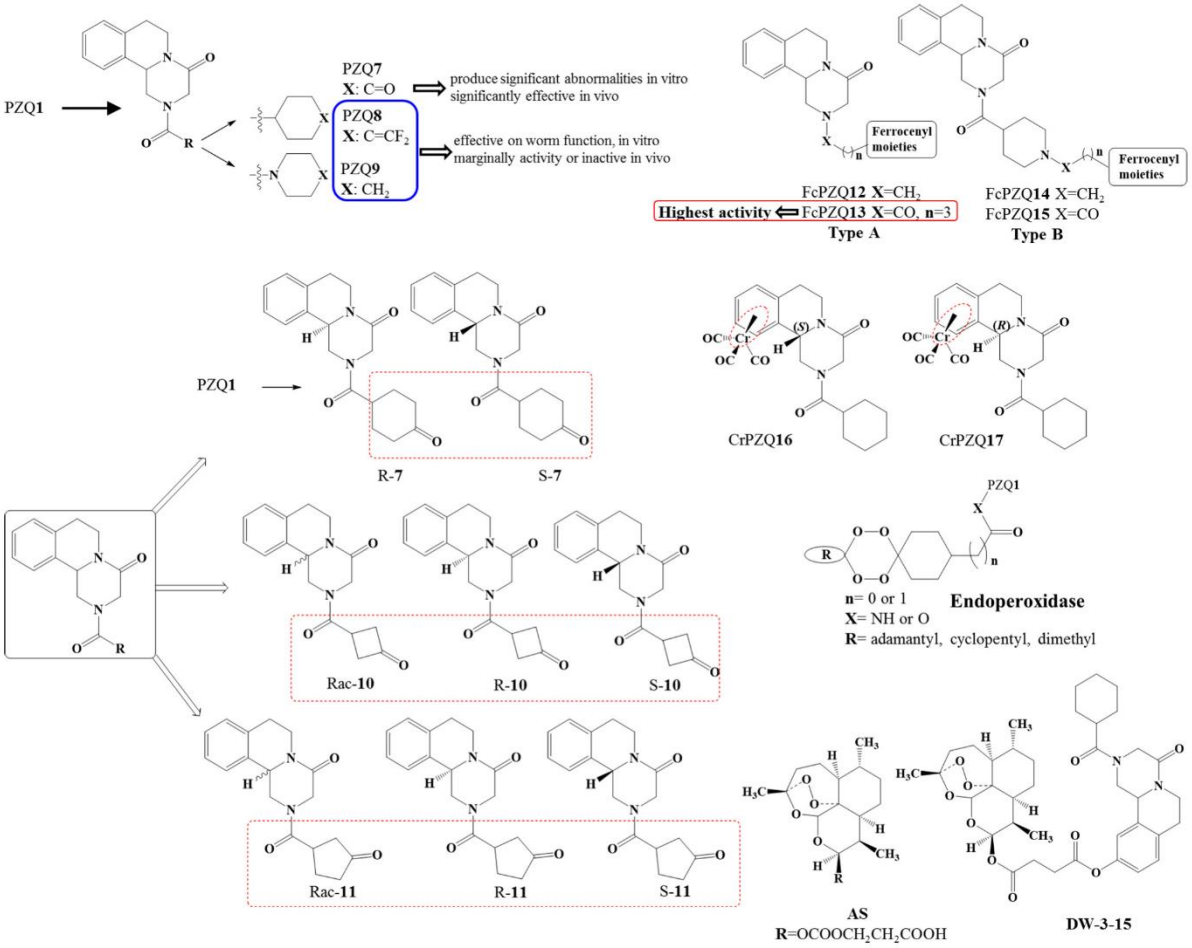

FIG 4 Structures of diverse PZQ derivatives developed and assessed for activity against schistosomes.

Moreover, potential action *in vitro* did not translate to impressive killing *in vivo* (64). The diminished activity of endoperoxidase derivatives might be associated with intrinsic aspects of their *in vivo* metabolism.

Apparently, the positions of chemical modifications played an important role in the compounds' activity. It seems that linkage through the metabolically liable cyclohexyl might not afford active derivatives, e.g., organometallic moieties. Moreover, PZQ derivatives have generally not achieved improved activity compared to that of the parent drug. Furthermore, in most cases, the promising *in vitro* activity of candidate drugs cannot be extrapolated to good *in vivo* activity since their pharmacokinetics and metabolic profiles are key determinants of their *in vivo* efficacy (53). Much remains to be done to develop an improved and effective derivative of PZQ1.

HOW DOES PZQ KILL SCHISTOSOMES?

Despite many years of use and the treatment of many millions of people, the mechanism(s) of action of PZQ1 has not been established yet. However, the early effects exerted by PZQ on the schistosome have been summarized under three main headings, (i) calcium influx into the whole parasite, (ii) muscle contraction, and (iii) surface modifications (65). It is tempting to link these phenomena into a single thread, assuming that calcium influx is the key event, which in turn induces muscle contraction and alterations to the tegument (65, 66). The correlation between increased intracel-

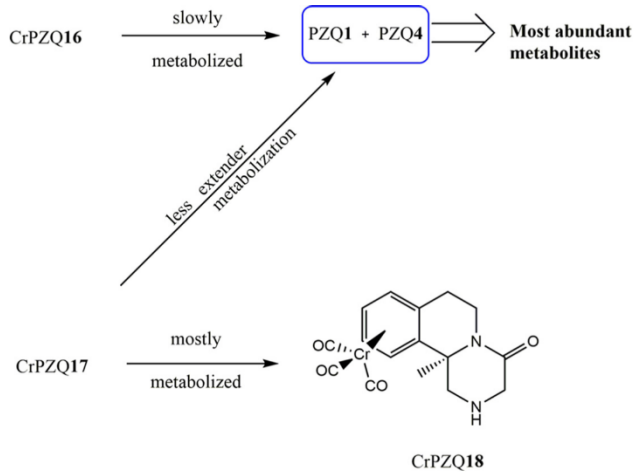

FIG 5 Metabolism of Cr-PZQ derivatives *in vitro* by human liver microsomes.

lular Ca^{2+} and muscular contractions in schistosomes exposed to PZQ has been known for decades. However, how PZQ1 disrupts homeostasis in schistosomes remains largely unknown. Diverse studies have focused on the phenomenon (Table 3). Initially, it was hypothesized that PZQ1 affects Ca^{2+} influx through voltage-operated calcium channels (67–73). However, in subsequent studies, it was shown that calcium accumulation by itself, as measured in parasites maintained *in vitro*, may not explain the schistosomicidal activity of PZQ1 (71, 72).

High-throughput transcriptomic approaches have been employed to address the refractory/susceptible nature of the developmental stages of schistosomes in terms of PZQ1 activity (73–77). These studies revealing genes that might be evolved in aerobic metabolism and cytosolic calcium regulation, suggesting that schistosomes undergo a transcriptomic response similar to that seen during oxidative stress (74). Moreover, it was demonstrated that CamKII (calcium/calmodulin-dependent protein kinase type II) appears to play a key role in the mode of action of PZQ1 and hence might be considered a promising novel drug target (76, 77). The use of mass spectrometry

TABLE 3 Key reports and findings focused on the mechanism of action of PZQ

Study design	Finding(s)	Reference(s)
Exposure of *S. mansoni* males *in vitro*	Muscular paralysis and rapid Ca^{2+} influx, removal of Ca^{2+} inhibited PZQ effect, tegument blebbing and disruption	67, 68
VOCC[a] β subunits expressed in *Xenopus oocytes*	Schistosome β subunits associated with drug sensitivity	69
Block VOCC, exposing schistosome to cytochalasin D	Inhibition of Ca^{2+} channels suppressed schistosomal activity	70, 71
Suppression of *Dugesia japonica* Ca^{2+} channel subunits by RNA interference	Suppression of Ca^{2+} channels of amputated parasite in two heads leads to inhibition of regeneration of two heads and tail	72, 73
Transcriptional response of *S. mansoni* to heat shock	>600 genes upregulated as possible targets of PZQ; schistosomes undergo oxidative-stress-like transcriptomic response	74
Gene expression in adult and juvenile *S. mansoni* cultured in PZQ	Juvenile schistosomes show enhanced transcriptomic elasticity	75
RNA interference-based silencing of CamKII	CamKII mitigated effect of PZQ by stabilizing Ca^{2+} fluxes within parasite muscles and tegument and might play role in mode of action	76, 77
Mass spectrometric characterization of surface lipids of schistosomes	Distinct chemical markers in female vs male responses to PZQ; PZQ may inhibit sphingomyelinase activity, impairing reproduction in females, whereas PZQ may impair activity of Na^+/K^+-ATPase in males	78, 79

[a]VOCC, voltage-operated calcium channels.

techniques revealed the existence of chemical markers that are distinct according to sex after drug exposure. Apparently, PZQ1 alters the conformation of the usual surface double lipid bilayer that surrounds schistosomes (78). Perhaps PZQ1 inhibits sphingomyelinase activity and thereby impairs reproduction by impeding the continuous release of eggs (79, 80).

Despite these efforts to understand how PZQ1 acts, the molecular targets remain elusive. Although from a medical point of view, how the drug acts might not be important as long as the drug is efficacious, the mechanism of action is relevant to improvement of the efficacy of new PZQ1 derivatives.

IS PZQ RESISTANCE IMMINENT?

Reliance on PZQ1 raises legitimate concerns about selection for PZQ resistance (65). MDA never reaches all of the infected people in a community, and so, the worm population remaining after treatment is not composed solely of resistant worms; there will still be a susceptible population that, in turn, reduces the likelihood of resistance (81–83). Whereas widespread drug resistance has not been proved, researchers have identified field and experimental isolates that exhibit significantly reduced susceptibility. These findings could portend the emergence of resistance to PZQ1 in schistosomes. Over the years, evidence of resistance to PZQ1 has been widely reviewed and remains controversial (81, 83–87). Moreover, the criteria used to classify a schistosome strain PZQ resistant are also controversial (81, 82). Here, we present an overview and synthesis of findings on this topic and also highlight potential mechanisms of drug resistance.

EXPERIMENTALLY INDUCED PZQ RESISTANCE

Attempts to induce resistance to PZQ1 in the laboratory were reported as early as the 1970s and have continued until the present, mostly focused on *S. mansoni* (88–90). Table 4 highlights some of the key studies (from our viewpoint) that attempt to demonstrate the appearance of resistance to PZQ. A hallmark study by Fallon and Doenhoff (91) published in 1994 demonstrated that *S. mansoni* developed resistance to PZQ1 over the course of several subcurative multiple doses of PZQ1 in mice; by the seventh generation of PZQ1 pressure, the population of schistosomes was 93% resistant to three PZQ1 doses of 300 mg/kg, a dose that killed 89% of the control schistosomes. Ismael and colleagues (92) studied the effect of PZQ1 at 300 and 500 mg/kg on successive generations of *S. mansoni* worms in mice and observed that at low subcurative doses, resistance to therapeutic doses of the drug appeared after several generations of the treatment regimen (92). More recently, Couto et al. (93) reported a novel method to induce resistance to PZQ1 in *S. mansoni*. Snails infected with schistosomes were treated successively with PZQ1 at 100 mg/kg for 5 consecutive days. Subsequently, mice were infected with cercariae released from the snails and treated with PZQ1 at 200, 400, or 800 mg/kg. This method is effective for inducing resistance of *S. mansoni* to PZQ1 in the laboratory and is far less costly and labor intensive than some other approaches mentioned above (93). Other studies have reported the generation of resistance to PZQ1 in *S. japonicum*, assayed in adult worms, cercariae, and miracidia (94). In contrast, we are not aware of reports describing experimental induction of resistance to PZQ1 in *S. haematobium*. Finally, it is also worth noting that the mode of action of the drug would be expected to be altered in strains that are insensitive to PZQ1 (81).

PZQ RESISTANCE IN THE FIELD

Reports of field resistance or therapeutic failure of PZQ are listed in Table 5. Most field surveys of resistance to PZQ1 focus on *S. mansoni*. Reduced susceptibility to PZQ1 has been widely found in foci of endemicity, notably in Africa, including Egypt and Senegal. An extremely low cure rate (18%) was reported in Senegal (95); however, it was suggested that failure of PZQ1 therapy occurs because of factors other than drug resistance, including very intense transmission and the presence of PZQ-refractory juvenile worms (immature parasites) (96). In Egypt, eggs obtained from treated and

TABLE 4 Key studies and findings focused on experimentally induced PZQ resistance in different *Schistosoma* species

Strain(s)	Study design	Treatment	Findings/outcomes	Reference(s)
S. mansoni WW and LE	Miracidia from feces of infected patients were used to infect *B. glabrata* to obtain cercaria (strain WW), which were used to infect mice; infected mice were treated with antischistosomal drugs; sensitivities of strains WW and LE to drugs were compared	Hycanthone, 80 and 20 mg/kg; niridazole, 100 and 50 mg/kg/day for 5 days; oxamniquine, 100 and 50 mg/kg, 1 dose	Hycanthone altered oogram pattern of 100% of mice infected with strain LE; hycanthone did not affect oogram pattern of mice infected with strain WW; strain WW was more resistant to niridazole and oxamniquine	88, 89
S. mansoni Brazilian	Mice were infected with *S. mansoni* obtained from infected individual, they were treated with different antischistosomal drugs during the time of embryological development of genital organs of schistosomula of both sexes	Oxamniquine, 50 mg/kg, 1 dose; oltipraz, 60 mg/kg daily for 5 days; PZQ, 50 mg/kg for 5 days	All treated groups had larger percentages of worms in liver and portal vein and significantly lighter parasite loads than control group, high rates of worm reduction, low rates of surviving worms, and 100% had changed oogram pattern; failure to induce resistance in Brazilian strain	90
S. mansoni Egyptian	*S. mansoni* infected mice were treated with subcurative different doses of PZQ after 6 wk p.i.;[a] eggs produced by worms that survived to treatment were used to infect snails	PZQ, 3 × 300 mg/kg	*S. mansoni* subjected to drug pressure may develop resistance to schistosomicidal drugs after relatively few passages; first demonstration of resistance to PZQ	91
	Miracidia obtained from eggs from infected patient were used to infected mice; eggs produced by worms that survive to treatment 6 wk p.i. were used to infect snails and mice of the following generations	PZQ, 300 or 500 mg/kg	Subcurative dose of PZQ led to development of resistance to therapeutic dose of PZQ in following generations	92
S. mansoni LE	Infected *B. glabrata* snails were treated with PZQ; after treatment, cercariae obtained from these snails (LE-PZQ isolate) and susceptible LE strains were used to infect mice that were treated p.i.	*B. glabrata*, 3 × 100 mg/kg, 5 consecutive days; infected mice, 45 days p.i., 200, 400, 800 mg/kg	Experimental model of development of resistance to *S. mansoni* using infected snails; mean no. of worms recovered from group of mice infected with LE-PZQ isolate treated with 200 and 400 mg/kg was significantly higher than that from mice infected with LE strain with same treatment; *in vitro*, worms of LE-PZQ isolates were also less susceptible to PZQ	93
S. japonicum	Mice were infected with isolates from two distinct regions, PZQ-susceptible isolates and PZQ-induced isolates, and then treated with PZQ; cercariae and miracidia of different isolates were exposed to PZQ solution, and morphological alterations were observed	Infected mice, 35 days p.i., 0,37.5,75, 150, 300, and 600 mg/kg; cercariae and miracidia, 10^{-5}, 5×10^{-6}, 5×10^{-7}, and 10^{-} M	PZQ-resistant isolates of *S. japonicum* were established in mice with subcurative doses of PZQ by artificial selection in laboratory; drug resistance might be exhibited by different developmental stages (miracidia, cercaria, adult worms); established PZQ ED_{50}s for different developmental stages	94

[a]p.i., postinfection.

uncured patients gave rise to schistosomes (*S. mansoni*) that showed 3- to 5-fold lower sensitivity to PZQ1 (97). In fact, *in vitro* measurements of PZQ1 susceptibility correlated well, in some cases, with the drug dose producing 50% of the maximal effect (ED_{50}), as determined in murine infections, further indicating that factors in the worms them-

TABLE 5 Key reports of field resistance or therapeutic failure of PZQ

Species	Country	Yr	Sample size	Treatment(s)	Outcome measure	Reference
S. mansoni	Senegal	1991	422	40 mg/kg	12 wk after treatment, cure rate only 18%, antigen detection positive in 90% of individuals; low cure rates may be due to intense transmission and/or development immune responses	95
	Egypt	1994	1,607	1 dose of 40 mg/kg, 3 successive doses of 40, 40 and 60 mg/kg	1–2.4% of villagers treated could not be completely cured of infection, and 3 of every 1,000 treated villagers may harbor parasites that can tolerate high doses of PZQ; extensive use of PZQ has not resulted in dramatic change in its efficacy	97
		2005	1,405		Compared with results obtained in 1994 in same villages, decreased prevalence and intensity of infections; first treatment resulted in normal cure rate (73–92%); after 3 successive doses, no uncured patients; drug failure did not increase over 10 years of therapeutic pressure in these villages	99
S. hematobium	Malawi[a]	1995	1	~40 mg/kg (30% was spit up)	3 doses of PZQ necessary to cure infection; concomitant *Giardia lamblia* infection might have caused malabsorption of drug; repeated courses of therapy may be necessary to cure infection, and both parasite and host factors should be considered if infection persists; 7 treatments necessary to eliminate eggs from parasites	100
	Senegal[a]	2006	2	40 mg/kg	Repeated standard treatment failed to clear infection	102
S. japonicum	Sichuan Province, China	1985	185	2 × 40 mg/kg	Low no. of treatment failures (only 1 remained infected); good compliance with treatment; PZQ remains effective against this schistosome	106
		2010	584		6 wk after treatment, 95% had no detectable eggs, 3% were still excreting eggs, and 2nd dose of drug was given; no detectable eggs were observed 6 wk after 2nd dose; efficacy of PZQ still high; no evidence of resistance detected	108
		2012	43	Single oral dose of 40 mg/kg, 30 mg/kg for 2 days	6 wk after second treatment, eggs not found were found in infected villagers; no evidence of resistance	110

[a]Travelers from this area of endemicity.

selves were responsible for the reduced susceptibility of these isolates to PZQ1 (98). Studies carried out 10 years later in the same area failed to show any hint of resistance to PZQ1 (99).

As noted, there is no evidence of *S. haematobium* resistance to PZQ1. However, some studies have reported failures of treatment to cure infections with this species (100–102). For example, Alonso et al. (102) described the case of two Spanish travelers with urogenital schistosomiasis in whom repeated standard treatment (a single 40-mg/kg dose of PZQ1) failed to clear the infection. Sabah et al. (103) hypothesized that people coming from areas where schistosomiasis is not endemic may lack an immunological component that has been shown to contribute to the activity of PZQ1 in experimental animals. Emergence of resistance of *S. japonicum* to PZQ1 has also received attention (101–105). However, despite large-scale and repeated use, the current efficacy of PZQ1 remains unchanged and it is highly effective at a curative dosage (a single dose of 40 mg/kg) in the main areas of China where schistosomiasis is endemic (106–108). Seto et al. (106) conducted a cross-sectional survey, in which the efficacy of PZQ1 was evaluated in 33 villages in Sichuan Province, where the prevalence of infection was found to be 5.7%. Of 3,269 persons tested, 185 were infected. The infected persons were treated two times with a 40-mg/kg dose of PZQ1, and only one remained infected, findings that support the notion that PZQ1 remains effective for the treatment of infection with *S. japonicum* in China.

Insensitive measurement of infection burdens may lead to overestimation of PZQ1

efficacy and thereby confound attempts to discriminate between reduced PZQ1 susceptibility and drug resistance. Diagnostic techniques for schistosomiasis are time-consuming, and many epidemiological assessments rely on microscopic observation of viable eggs in urine (*S. haematobium*) and feces (*S. mansoni*, *S. japonicum*) (109, 110). However, fluctuation of egg output in urine or stool occurs routinely, negatively influencing the sensitivity of the assay in the absence of repeated sampling (111). New diagnostic techniques such as egg detection by PCR aim to improve sensitivity, but the sampling limitations persist (112, 113). Despite the development of new tools for diagnosis (reviewed in reference 114), there remains a need for better diagnostics, both in the field and in the clinic. In addition to the importance of improvements for clinical diagnosis, advances in diagnostic tools are also critical in programs targeting elimination by MDA and for the development and assessment of new drugs and vaccines (3).

MECHANISM OF PZQ RESISTANCE

In the absence of the exact mechanism of action of PZQ1, the mechanism of drug resistance in schistosomes also remains unclear (115). However, the likely nature of the mechanism of PZQ resistance has been described, such as induction of ATP-binding cassette (ABC) transporters (ABC transporters are proteins involved in the transport of toxins and xenobiotics). Several members of this family, like P-glycoprotein (Pgp) and multidrug resistance (MDR)-associated proteins (MRPs) represent two classes of these MDR transporters (116, 117). ABC transport protein homologues from *S. mansoni* are known, i.e., SmMRP1 (orthologue of MRP1) and SMDR2 (orthologue of Pgp) (106). Juvenile schistosomes express ~2.5-fold higher basal levels of SMDR2 and SmMRP1 than adults, higher levels of SMRD2 RNA are seen in females than in males, and higher SmMRP1 levels are seen in males than in females (118). Furthermore, SMRD2 is modulated by PZQ1, suggesting that PZQ1 is also a substrate for SMRD2 (119). Transcriptomic analysis reveals increasing levels of transcripts encoding the ABC transporters SMDR1, SmMRP1, SmMRD2, and SMDR3 in juveniles exposed to PZQ1 *in vitro*, supporting the notion that ABC transporters participate in resistance to PZQ1 in schistosomes (75). Guglielmo et al. (120) developed a series of PZQ NO-donors furoxans that are worthy of investigation in view of their potential activity against PZQ-resistant schistosomes. Involvement of Ca^{2+} channel changes in resistance to PZQ has been widely described (121). Nonetheless, whether these phenomena are responsible for drug action or represent downstream consequences has not been established (83, 122).

CONCLUDING REMARKS

Because of its efficacy, safety, cost, and indeed the lack of alternatives, PZQ1 has remained the drug of choice for schistosomiasis treatment and transmission control for >40 years (15, 16). Yet PZQ1 has drawbacks, including inactivity against juvenile schistosomes. Moreover, reliance on a single drug for the treatment of a disease with the global public significance of schistosomiasis risks facilitating the development and spread of drug resistance, especially since reduced susceptibility has occurred frequently both in the field and in the laboratory. A pressing need for new interventions has arisen, including novel compounds with modes of action discrete from those of PZQ1 and methods to detect the appearance and spread of resistance to PZQ1 (123). Despite the novel structures of several derivatives of PZQ1, most are sufficiently efficacious to warrant closer investigation in clinical trials. In addition, understanding the mechanism of action of PZQ1 and its metabolism is critical since this information would facilitate the elucidation of novel targets and/or lead to improvements in the efficacy of this essential and singular medicine.

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Appendix 3

Drug repurposing for schistosomiasis: combinations of drugs or biomolecules

pharmaceuticals

MDPI

Review

Drug Repurposing for Schistosomiasis: Combinations of Drugs or Biomolecules

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Abstract: Schistosomiasis is a major neglected tropical disease. Control of schistosomiasis currently relies on a single drug, praziquantel, and despite its efficacy against the all schistosome species that parasitize humans, it displays some problematic drawbacks and alone is ineffective in counteracting adverse pathologies associated with infection. Moreover, due to the development of the potential emergence of PZQ-resistant strains, the search for additional or alternative antischistosomal drugs have become a public health priority. The current drug discovery for schistosomiasis has been slow and uninspiring. By contrast, repurposing of existing approved drugs may offer a safe, rapid and cost-effective alternative. Combined treatment with PZQ and other drugs with different mode of action, i.e., antimalarials, shows promise results. In addition, a combination of anthelminthic drugs with antioxidant might be advantageous for modulating oxidative processes associated with schistosomiasis. Herein, we review studies dealing with combination therapies that involve PZQ and other anthelminthic drugs and/or antioxidant agents in treatment of schistosomiasis. Whereas PZQ combined with antioxidant agents might or might not interfere with anthelminthic efficacy, combinations may nonetheless ameliorate tissue damage and infection-associated complications. In fact, alone or combine with other drugs, antioxidants might be a valuable adjuvant to reduce morbidity and mortality of schistosomiasis. Therefore, attempting new combinations of anthelmintic drugs with other biomolecules such as antioxidants provides new avenues for discovery of alternatives to PZQ.

Keywords: schistosomiasis; drug repurposing; praziquantel; antioxidants

1. Introduction

The Tropical Diseases Research arm of the World Health Organization classifies schistosomiasis as one of the major neglected tropical diseases. It is considered the most important of the helminthoses of humanity in terms of mortality and morbidity [1,2]. Schistosomiasis is endemic in 70 countries worldwide, affecting >250 million people [3–5]; yet this number likely is an underestimate, with recent studies documenting infection of 391–597 million people, with 800 million, mostly children, at risk of infection [6,7], and people are infected during domestic, recreational and occupational water [8,9].

The infectious agents of human schistosomiasis include three major species; *Schistosoma mansoni* and *S. japonicum* which cause hepato-intestinal schistosomiasis in Africa, the Middle East, South America and the Caribbean whereas *S. haematobium*, endemic in Africa and the Middle East and is responsible for urogenital schistosomiasis (UGS) [10,11]. Infection with *S. haematobium* also is classified as a group I carcinogen; UGS often leads to squamous cell carcinoma of the bladder [12]. Although schistosomiasis is generally restricted to the tropics and sub-tropics, a recent outbreak in Corsica demonstrates the potential for reemergence of this infectious disease in new, economically developed regions in southern Europe [13].

Infection follows exposure to freshwater containing free-swimming larval forms of the parasite (this larva is termed the cercaria). Cercariae penetrate intact human skin, where the larva sheds its tail and which the larva, now termed the schistosomulum, enters the bloodstream which travels via pulmonary artery to the lungs. After exiting the lungs, schistosomula re-enter the venous circulation and circulates for several weeks, until the adult schistosomes take up residence within the mesenteric veins (*S. mansoni* and *S. japonicum*) or the vesical plexus and veins that drain the ureter and nearby pelvic organs (*S. haematobium*) [3] where the worms mate, and commence egg laying about 5–7 weeks post-infection [3,14,15]. Schistosomes are long lived in these sites, often for decades, and they shed large numbers of the eggs each day. The eggs must transverse the walls of the blood vessels with the goal of reaching the lumen of the intestine or bladder to be excreted or evacuated in urine or feces, respectively [3]. However, many eggs become entrapped in the tissues and organs including the walls of the bladder, bowel, and in the liver which comes with blood of return to this organ [14,15]. The developmental cycle of the parasite is completed when eggs reach freshwater, hatch, and release the miracidium, a ciliated larva, which seeks out the aquatic snail. Following infection of the snail, the miracidium transforms into the sporocyst stage of the schistosome. The cercariae develop within the two generation of sporocyst (I and II), and are eventually released from the snail into the water, completing the developmental cycle [3].

Control strategies employ approaches to block transmission and reduce the disease burden including mass and targeted chemotherapy, and absence of safe water and sanitation facilities, modification of the environment, and use of molluscides [3]. The goal of these approaches includes mitigating the burden of disease, by reducing morbidity [16] at both the individual and community levels [17]. This review presents and discusses a new perspective of current approaches for treatment of schistosomiasis. Combination treatments with PZQ and other anthelminthic drugs as well as administration of antioxidant agents alone or as adjuvant in treatment of schistosomiasis are addressed and reviewed.

2. Praziquantel: Mainstay Chemotherapy against Schistosomiasis

The pyrazino-isoquinolone compound praziquantel (PZQ, Figure 1) is widely accepted and used for the treatment of all forms of schistosomiasis and, indeed, infections with most flatworm parasites [18,19]. PZQ is effective against all schistosome species and generally causes only mild and transient side effects [17]. Since 2006, many millions of doses of PZQ have been consumed, mostly in Sub-Saharan Africa; and it has been estimated that by 2018 as many as 235 million people will have been treated with PZQ [20]. Nonetheless, PZQ presents some drawbacks such as is distributed as racemate that includes equivalent proportions of biologically active *R*-PZQ and inactive *S*-PZQ enantiomers (Figure 1), consequently half of PZQ dose is pharmacology inactive; low solubility and passes through extensive metabolism via hydroxylation of the absorbed drugs to inactive metabolites

[21,22]. The presence of *S*-PZQ, the inactive diastereosisomer, both contribute to the large size of the tablet and its bitter taste, which impedes compliance by patients, especially children [21,23,24]. Another major drawback of PZQ is its inefficacy against juvenile stages relative to mature worms, thus complete cure is unusual with a single dose of PZQ [22,25,26].

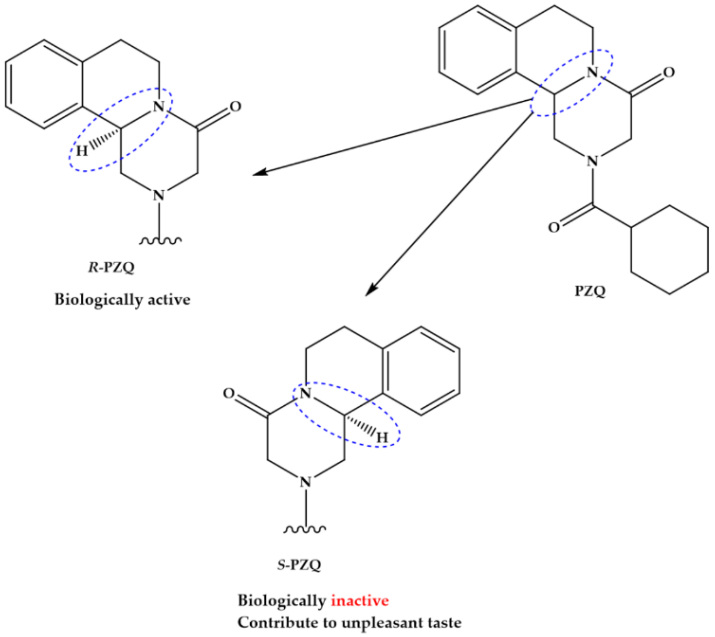


Figure 1. Chemical structures and characteristics of praziquantel and its enantiomers, *R*-PZQ and *S*-PZQ.

In spite of the comprehensive use of PZQ, the model of action against schistosomes and targets molecules of PZQ in schistosomes remain to be elucidated. Substantial evidence implicates calcium channels as targets because schistosomes display marked fluxes in calcium ion (Ca^{2+}) following exposure to PZQ [27], that leads to tetany of the musculature [28] accompanies by damage to the tegument [29]. The hypothesis that PZQ perturbs Ca^{2+} channels is supported by findings of studies that employed calcium-channels blockers and cytochalasin D [30]. More recently, was demonstrated that PZQ is a GPCR ligand, with *R*-PZQ acting as an agonist at human 5-HT_{2B} receptor [31].

Reliance for many decades on singular drug has obviously raised legitimate concerns about PZQ-resistance [32]. Whereas widespread drug resistance to PZQ has not arisen, field and experimental isolates that exhibit significantly reduce susceptibility have been described, which foreshadows for emergence of resistance against parasites [33–35].

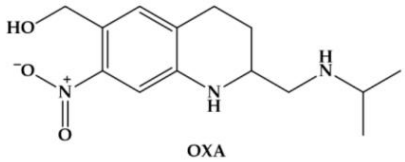
Despite the efficacy of PZQ in reducing morbidity, the reliance of treatment and control on this drug and the concerning with emergence of resistance, prompt the search for additional therapeutics or other control strategies [35–38]. Continuous efforts have been made in order to circumvent reliance on a single medicine through synthesis of derivatives of PZQ and investigation of their antischistosomal activity [39,40]. Unfortunately, however, these derivatives have generally not achieved improved activity compared to that of the parental drug. In most cases, the promising in vitro activity of candidate drugs cannot be extrapolated to accept in vivo activity, likely related to pharmacokinetics and metabolic profiles, the key determinants of in vivo efficacy [32]. Several morbidity control strategies are often combined, but they need to be improved for comprehensive integrated control programs. In addition, given the reliance on PZQ, it is necessary optimize the useful life span of PZQ, through investigation of the potential for administration of combined PZQ therapies, avoiding the emergence and evolution of drug resistance. Over the years, it has been emphasized that this strategy would reveal synergistic effects and therefore might improve the treatment of helminth infections.

3. Treatment of Schistosomiasis: Anthelminthic Drugs Alone or Combined

Rational combination chemotherapy was developed for tuberculosis and other bacterial infections [41]. It is used for chemotherapy of cancer and acquired immune deficiency syndrome (AIDS) [42], and for malaria [43,44]. The main aims of this therapeutic strategy are to achieve additive/synergistic therapeutic effect and to minimize or delay the appearance of drug resistance [41–43]. When synergism/additive effect is exhibited, achieving similar or even enhanced efficacy at lower doses can be expected, along with reduction in side effects [42]. In the case of schistosomiasis, ideally the associated drugs would exhibit divergent mechanism of action to PZQ and/or target the immature schistosomes to enhance cure and egg reduction rates, as well as pathologies associated with infection. Herein, we summarize the evidence from experimental studies, in vitro and in vivo, as well as human clinical trials involving combination of different anthelmintic drugs against schistosomiasis. Initially, we start to introduce the drugs used against the disease and their combination in experimental studies (in vitro and in vivo) as well human clinical trials.

3.1. Oxamniquine

Until recently, oxamniquine (OXA) (Figure 2) was the drug of choice for *Schistosomiasis mansoni* for many decades in Brazil [45].



OXA

Figure 2. Chemical structure of oxamniquine (OXA), an antischistosomal drug used against *Schistosomiasis mansoni*.

The efficacy of OXA is confined to *S. mansoni*, unlike PZQ which is active against all schistosomes. However, like PZQ, OXA is more active against adult worms than the juvenile stages; and males are considerably more susceptible than females [46]. OXA induces less specific morphological alterations on parasite, and its hepatic shift occurs much more slowly post treatment. It has been considered that mode of action of OXA is related to inhibition of the nucleic acid metabolism. The hypothesis relies on activation of OXA by a single step, in which a schistosome enzyme converts the drug into ester that spontaneously dissociates, resulting in electrophilic reactants capable of alkylation of schistosome DNA [16]. This drug is also converted to a reactive sulfate ester and the activating enzyme is a sulfotransferase [47]. Resistance in *S. mansoni* to OXA is controlled by a single autosomal recessive gene. Anderson coworkers demonstrated, using two strains of *S. mansoni* from Brazil that differed 500-fold in sensitivity to OXA, and by Mendelian-type genetic crosses followed by linkage mapping, genomic sequencing and X-ray crystallography that a schistosome gene that encodes a sulfotransferase responsible for activating the prodrug to its active form, which in turn intercalates into the genome and interrupts nucleic acid synthesis [48].

As PZQ, OXA is safe and side effects are limited to mild but transient dizziness [49]. Although, low cure rates obtained for *S. mansoni*-infected patients treated with OXA have been described repeatedly, thus far, these do not constitute a significant concern for public health [15].

Combination therapy with OXA and PZQ have been used since 1980s, both in the laboratory and the clinic. However, findings with this combination are not clear, and would benefit with further investigation and stricter criteria [50]. In 1983, Shaw and Brammer investigated combinations of PZQ and OXA in adult *S. mansoni* in mice [51]. The findings were encouraging with respect to enhanced antischistosomal effects, more marked than either drug alone [51]. This combination regimen of low doses (1/3 curative doses of both drugs) was more effective four hours post-infection (p.i.), while administration at 5 weeks p.i. resulted in more marked worm burden reduction than PZQ and OXA monotherapy [52]. These results might be related to an early effect on the developing gonads of the schistosomula when mice were treated with OXA 4 h p.i. and interference with copulation when PZQ

was given 5 weeks p.i. [52]. In a different study, low dose combinations of OXA-PZQ were active on different strains of *S. mansoni*—Venezuelan YT and SM and Brazilian BH, Complete elimination of all three strains was accomplished. When given alone, high doses of OXA or PZQ were required to obtain similar efficacy obtained with combined low-dose formulations. From these reports, it is reasonable to conclude that a combination of OXA-PZQ acts through synergism [53].

Human clinical trials were undertaken to evaluate efficacy of this combination during combination treatment in 158 schoolchildren aged 6–20 infected with *S. mansoni* in Malawi [54]. Findings from 102 children with *S. mansoni* infection and 56 children with *S. haematobium* infection were reported. Significant egg reduction (93–99%) was observed in *S. mansoni*-infected children treated with PZQ (15 to 20 mg/kg) and OXA (7.5 to 10 mg/kg). Combined treatment also markedly reduced numbers of eggs of *S. haematobium* passed in urine (97–99.2%), somewhat unexpectedly since OXA is generally considered not active against this schistosome species [54]. Some authors considered that several aspects of this trial may have influenced these unusual findings including (1) small number of individuals in the treatment groups; (2) co-infection with both parasites of half the participants; (3) the use of only egg counts as the only end point; (4) high infection intensities in the population, and (5) the collection of only one stool or urine sample specimen at one month post-treatment [15]

A similar clinical trial was conducted in children in Zimbabwe, which included 58 school age participants who were infected with *S. mansoni* and *S. haematobium*. PZQ (20 mg/kg) and OXA (10 mg/kg) achieved a cure rate of 89% for the *S. mansoni* infections [55]. Here the PZQ-OXA combination failed to cure *S. haematobium* infections with high egg count reduction found for both parasite species being attributed to PZQ in cases of *S. haematobium* infections [55]. The study design exhibited limitations (small sample size, among others) similar to those carried out in Malawi, although the study end points included both cure and egg reduction rates. In addition, multiple samples of feces and urine was examined in order to assess therapeutic efficacy [54], and accordingly the findings may be more solid than those from the clinical trial in Malawi.

3.2. *Antimalarials*

Antimalarials have been tested against schistosomiasis either alone or combined. Figure 3 depicts the chemical struct of some of these compounds.

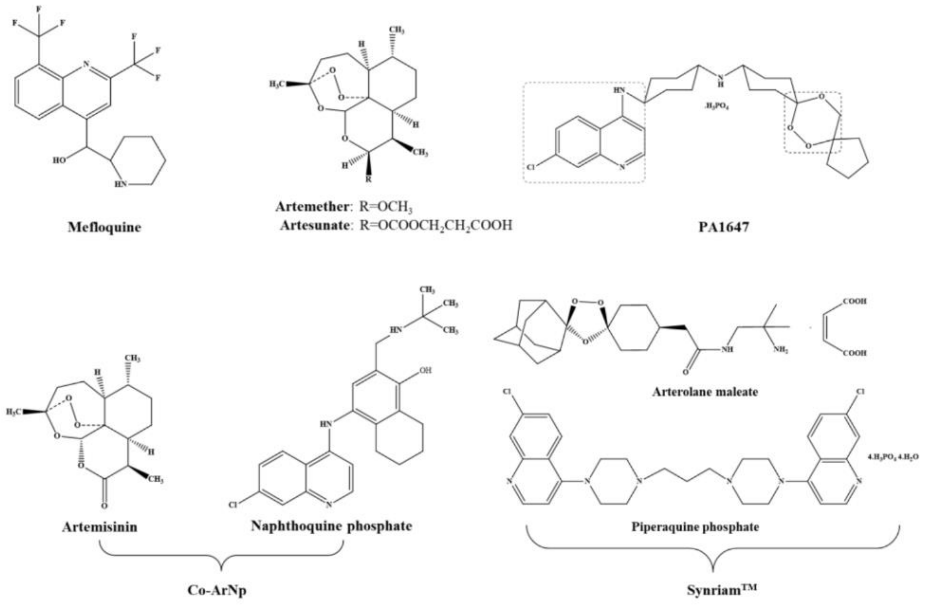


Figure 3. Antimalarials studied alone or in combined regimen on experimental infections and clinical trials. PA1647 and Synriam™ are trioxaquines and trioxalanes respectively. Note that PA1647 contain two antimalarial pharmacophores: a 4-aminoquinolone and a 1,2,4-trioxane.

3.2.1. Artemisinins Derivatives

In searching for alternatives to PZQ, studies have been undertaken with semi-synthetic derivatives of the sesquiterpene lactone, artemisinins, including artemether (ART) and artesunate (AS) (Figure 3) which possess activity against the human schistosomes [15]. Dissimilar to PZQ and OXA, ART and AS exhibits high levels of activity against juveniles while the invasive stages and adult worm are less susceptible. Moreover, unlike OXA, adult female worms are somewhat more susceptible to ART than male worms [55]. Although the exact mechanism of action of artemisinin against schistosome remains elusive, evidence suggests that ART alters glycogen content in schistosomes [56], accompanied by morphological alterations similar to those induced by PZQ [55]. Exposure of schistosomes in medium containing ART plus hemin kills the worms while exposure of compounds alone does not. Hence, ART might be activated by hemin which subsequently cleaves an endoperoxidase bridge to liberate free radicals which, in turn, form covalently bind to schistosome proteins [57]. Also, hemin enhances in vivo efficacy of ART against *S. mansoni* [58]. Artemisinins are not only safe [59], but also possess a discrete mode of action of PZQ [60]. In fact, in a recent meta-analysis it was confirmed that artemisinins used in combination with PZQ have the potential to increase the cure rates in schistosomiasis treatment [61].

Arthemeter

Efficacy of PZQ-ART combinations were assessed in performed in different host-parasite models [62,63]. The reports revealed consistently higher worm burden reductions following treatment with combined regimen compared to PZQ or ART alone. In rabbits infected with juvenile and adult *S. japonicum,* treatment with 50 mg/kg of PZQ and 10 mg/kg of ART with one day apart significantly reduced worm burden (82%) compared with PZQ and ART monotherapies (66% and 44%, respectively) [62]. Similar results were seen with rabbits infected only with adult *S. japonicum* [63]. Hamsters infected with juvenile and adult *S. mansoni* were simultaneously treated with 75 mg/kg of PZQ and 150 mg/kg of ART. Administration of the combined regimen reduced worm burden by 77% which was significantly higher than 2% reduction achieved with PZQ monotherapy, but, it was not significantly different from 66% reduction obtain with ART alone [64]. Mahmoud and Botros [64] investigated the therapeutic effect of PZQ-ART combination in mice infected with *S. mansoni* in differential developmental stages. The antischistosomal effect of a single dose of ART was similar for adult and juvenile *S. mansoni*. Histopathological changes were evaluated. In contrast to results observed in hamsters, administration of PZQ-ART combination led to >90% worm reduction which was not statistically significant when compared with 95% achieved with PZQ monotherapy. Nonetheless, the impact of combined treatment regimen on *S. mansoni* eggs was impressive, with complete absence of eggs from tissues with minor histopathological changes in the liver. Although the few residual worms recovered from groups receiving the PZQ-ART combination were almost sterile and incapable of oviposition, this evidence alone might not explain the complete absence of eggs and granulomas from tissues [64]. Free radical liberation plays a role in drug induced-immune responses [64].

The safety and efficacy of ART-PZQ in different regimens for treating schistosomiasis japonica was assessed on a randomized double-blind, placebo controlled clinical trial in 196 Chinese patients for a 45-day period [65]. Administration of PZQ either or without ART during acute schistosomiasis was highly efficacious. Two end-points were included in trial, infection status (determined by stool examination) and blood chemistry. The combination of PZQ-ART in two distinct dosages (60 mg/kg and 120 mg/kg of PZQ plus 6 mg/kg ART) achieved parasitological cure rates of 98.0% and 97.7% ($p > 0.05$), respectively. Nonetheless, these results were not statistically different from those obtained in control group (PZQ/placebo, 96.4% and 95.7%, $p > 0.05$) [65]. Apparently, in contrast to laboratory findings with rodents, the combination of ART-PZQ did not improve the efficacy in infected people, when compared to PZQ alone.

Artesunate

Although AS caused a significant reduction from egg tissue in comparison to PZQ, curiously, AS did not markedly reduce numbers of female parasites. AS appeared to impair fecundity of the adult females rendering them sterile rather than limited release of eggs [66]. Administration AS-PZQ to mice infected with *S. mansoni* significantly reduced in total worm count with complete eradication of female worms and tissue egg count in comparison to monotherapy with PZQ or AS. The action of the AS-PZQ combination may relate to the effect of AS in adult females, leading to a reduction of eggs with efficacy of PZQ against adult worms that results in elimination of the worms. In addition, Abdin and co-authors investigated effects of AS-PZQ on schistosome thioredoxin glutathione reductase (TGR) and cytochrome c peroxidase (CcP). They suggested that AS activity might be mediated by expression of these genes; by contrast, PZQ failed to influence expression of these genes. The loss of these two defensive enzymes likely renders the parasite vulnerable during its different stages to reactive oxygen species (ROS) [66]. Although CcP, a mitochondrial enzyme expressed in adult schistosomes that protects against endogenous and exogenous H_2O_2, it is unlikely that CcP exerts a general effect on peroxidation outside the mitochondrion [66]. Both CcP and TGR might be developed as drug targets since TGR especially displays functional and biochemical differences between redox metabolism and the human host [66,67].

Efficacy of a AS-PZQ combination was evaluated in a non-blinded open-label trial in Senegal that enrolled 110 local residents who were stool-positive for *S. mansoni* infection, aged 1 to 60 years. These participants were assigned groups that received either a single oral dose of PZQ (40 mg/kg), the recommended dose regimen of AS (4 mg/kg followed by four daily doses of 2 mg/kg), or a combined treatment. Parasitological parameters including cure and egg reduction rates were evaluated at 5, 12 and 24 weeks following treatment using two Kato-Katz thick smears taken from the same, single stool specimen. Since Senegal is an area of intense transmission of *S. mansoni*, reinfections occur frequently and quickly. In this regard, the therapeutic efficacy during first 5 weeks will be discussed here. Despite treatment with AS-PZQ resulting in cure and reduced numbers of eggs higher (69% and 89%, respectively) than with monotherapy, the egg reduction rate was similar to PZQ alone (84%), however, it was higher than AS alone (59%) [68]. AS also failed to affect the number of eggs.

A combination of AS-PZQ was evaluated in a double-blind, randomized, placebo-controlled study in Gabon that enrolled 296 children aged 5 to 13 infected with *S. haematobium*. By 8 weeks post-treatment, egg numbers in urine in two consecutive urine samples were ascertained. A cure rate of 81% (95% confidence interval, CI) was observed in the group treated with combined AS-PZQ which is not significant when compared to the cure rate for PZQ monotherapy (73%, 95% CI). In addition, the cure rate of 27% (95% CI) obtained in the AS monotherapy group was also not significantly different to the placebo (20%, 95% CI), which was attributed to day-a-day variation in numbers of eggs in the urine [69].

Administration of AS-PZQ was assessed in the treatment of urogenital schistosomiasis (UGS) in several villages of Nigeria [70,71]. Inyang-Etoh and colleagues enrolled 312 randomly selected schoolchildren aged 4 to 20 years. Groups were treated with PZQ-placebo, AS-placebo, PZQ (40 mg/kg), AS (4 mg/kg), or a combination of PZQ (40 mg/kg) and AS (4 mg/kg) [70]. Cure and egg rate were assessed by examination of urine for schistosome eggs. As observed in Gabon [69], high cure and mean ova reduction (88.6% and 93.6%) rates were obtained with the combination of drugs while PZQ achieve a cure rate 72.7% and AS 70.5%. However, significant differences were not apparent among cure rate with AS-PZQ, and PZQ with or without placebo. These results reinforce that PZQ is maximally active again adult schistosomes. The differences between cure rates with PZQ-AS compared to the AS-placebo support the notion that AS has fewer schistosomicidal activity against adult schistosomes [70]. Similar findings were seen in a nearby village among 70 children diagnosed with UGS following administration of the AS-PZQ combination, where treatment with a combination of AS (4 mg/kg/day over 3 days) plus PZQ (40 mg/kg once) and single oral dose of PZQ (40 mg/kg). Number of eggs in urine one month following drug administration were ascertained. The AS-PZQ combination lead to significantly higher cure (85.7%, 95% CI) compared to monotherapy with PZQ

(51.4%, 95% CI). These results are consistent with those described above and might be attributed to a synergistic effect of these two drugs, but using a different mode of action [71]. Cure rates with the same combined therapy differed among villages, which may have reflected divergent susceptibility profiles of parasite genotypes across the endemic range of schistosomiasis.

Artesunate Combined with Sulfamethoxypyrazine/Pyrimethamine

The antischistosomal activity of a new combination therapy AS-sulfamethoxy-pyrazine/pyrimethamine (AS-SMP) was evaluated among 800 school-aged children infected with *S. haematobium* [72]. Children were allocated into groups and treated with PZQ alone and AS-SMP, and urine samples were examined on days −1, 0, 28 and 29. A higher cure rate was achieved with PZQ group (53%), suggesting that AS-SMP (43.9%, $p = 0.011$) is not effective at least against adult worms or even with this schistosome species. A moderate enhancement of egg reduction was seen in PZQ groups (95.6%) in comparison to AS-SMP (92.8%, $p = 0.096$) [72]. This might relate to the fact of the dose administered corresponded to doses used for malaria. Additionally, the study revealed that safety and tolerability profiles of this combination were similar to PZQ [72]. It will be necessary assess if increasing the dose would enhance the antischistosomal efficacy and retain safety and tolerability. Efficacy and safety of AS-SMP should be evaluated for the other schistosome species [72].

Artemisinin and Naphthoquine Phosphate

A novel oral antimalarial drug, naphthoquine consisting in a combination of naphthoquine phosphate and artemisinin (Co-ArNp) (Figure 3) exhibits activity against *S. mansoni* in vivo [73]. The nature of the specific pharmacodynamic interaction between artemisinin and naphthoquine phosphate in the formulation was not clear, however co-administration of both drugs induced significant synergistic interaction [73]. Oral administration of Co-ArNp in a single dose of 400 mg/kg in mice infected with *S. mansoni* (Egyptian strain) on day 7 p.i. reduced the worm burden by 95%. Increasing the oral dose up to 600 mg/kg on day 21 p.i. resulted in an elimination of all female worms before they commenced laying eggs. In addition, the combined regimen provided significant reductions in the hepatic and intestinal tissue egg loads, and induced significant alterations in oogram pattern. These alterations might be attributed to the activity of artemisinin, which might be augmented by co-administration with napthoquine phosphate harming the female worms and oviposition [74]. Despite these promising findings in mice, it was necessary to study the toxicity of this combination. Clinical trials will be required to determine whether artemisinin/PZQ combination therapy offers advantages and whether the inevitable higher cost of such a combined treatment makes it practicable, and artemisinins (alone or in combination with PZQ) to treatment of schistosomiasis is the risk of driving artemisinin resistance in malaria in areas where both diseases are endemic [75].

3.2.2. Mefloquine

Mefloquine (MFQ, Figure 3), an antimalarial agent, is considered one of the best antischistosomal drugs [76]. Similar to artemisinin, MFQ is also active against developmental stages of schistosomes. In adult worms, MFQ induced extensive, severe damage on tegument, musculature, digestive and reproductive systems [76–78]. It was anticipated that combinations of MFQ with other drugs would be more effective rather PZQ monotherapy. Therefore, evaluation of combine regimens of MFQ-PZQ and MFQ-artemisinin derivatives against schistosomiasis have been performed both experimentally (*in vitro* and in vivo) and in human clinical trials [79–84].

The efficacy of MFQ administered orally at single, multiple doses, or in combination with AS, ART, or PZQ was assessed in the *S. japonicum*-mouse model at 4 weeks post-treatment. Administration of MFQ (50 mg/kg or 100 mg/kg) in combination with ART or AS (100 mg/kg) totally eliminated female worms, especially in mice treated with combination of MFQ-ART. The better results were achieved using higher doses of 100 mg/kg. Elimination of females might be a valuable target since eggs are central of the pathogenesis of schistosomiasis [79]. Also, combined regimens

achieved a significant decrease of total worm burden (76.7% for MFQ-AS, 1:1, 100 mg/kg and MFQ-ART, 1:1, 100 mg/kg: 87.8%) compared to monotherapies (AS 100 mg/kg: 59.8% versus MFQ 100 mg/kg: 67.9% and ART 100 mg/kg: 55.6%). This outcome suggests a synergistic effect between MFQ and artemisinin derivatives [79]. MFQ-PZQ was also evaluated in *S. mansoni* infected mice at the same doses as above and also at 400 mg/kg each. Higher total and female worm burden reductions (86.0% and 93.0%, respectively) were achieved only when either MFQ-PZQ at the highest dose were given simultaneously or MFQ was given 24 h prior to PZQ. At the lower doses of 50 and 100 mg/kg, combinations involving PZQ followed a day later with MFQ induced only moderate total worm burden reductions, 47.8–54.7%. The most impressive outcome was seen when PZQ treatment were followed by MFQ, which suggests that PZQ might play a role in the antagonistic effects when it was administered before MFQ [80]. In mice simultaneously infected with *S. mansoni* 14-day-old schistosomula and 49-day-old adult worms, administration of a daily dose of 100 mg/kg of PZQ and MFQ for two consecutive days markedly reduced worm burdens of immature and adult schistosomes (both >95%) as well as immature eggs in comparison to monotherapies (PZQ: 29.2% and 49.6%; MFQ: 41.9% and 67.4%, respectively). Additionally, histopathological examination on liver sections revealed that the combined regimen significant reduced granuloma diameter in comparison to monotherapies. Enhanced worm burden reduction might be correlated with healing of hepatic granulomatous lesion, since eradication of females led to inhibition of oviposition [81]. Abdlel-Fattah and colleagues obtained contradictory results from those described above. In *S. mansoni* infected mice treated 3 or 6 weeks with curative (400 mg/kg MFQ and 500 mg/kg PZQ) or subcurative (200 mg/kg MFQ and 250 mg/kg PZQ) doses, MFQ monotherapy was more potent than the combined regimen in full dose regimen and PZQ monotherapy in its low regimen [82]. The authors attributed the additive/synergistic effect of PZQ and MFQ, observed from other studies [80,81] to the simultaneous presence of juvenile and adult worms, a difference that might explain the failure to achieve an additive effect in other studies [82].

MFQ-PZQ combinations were evaluated in clinical trials. A randomized, exploratory open-label trial was carried out in Cote d'Ivoire where 83 schoolchildren infected with *S. haematobium* were divided in four groups and treated with: (i) MFQ (single dose of 25 mg/kg); (ii) AS (4 mg/kg daily for 3 days); (iii) MFQ-AS (single dose of 40 mg/kg) and iv) PZQ (single dose 40 mg/kg). PZQ achieved highest cure rate (83%) followed by combine regimen (61%) while monotherapies of MFQ and AS only resulted in lower cure rate (21% and 25%). In children, concurrently infected with *S. haematobium* and *S. mansoni*, the treatment with PZQ and MFQ-AS resulted in high cure rates of 83% and 75% and egg reduction rates, 97% and 96% respectively. Despite the higher cure rate with PZQ monotherapy, the combination of MFQ-AS, administrated in accordance with currently recommended malaria treatment, showed encouraging results in co-infected children [83]. Keiser et al. assessed the efficacy and tolerability of similar treatment described, with the inclusion of MFQ-AS (3 × (100 mg/kg AS + 250 mg/kg MFQ)) combined with PZQ (MFQ-AS-PZQ) [84]. Urine from 61 children was collected before, and on days 21–22 and 78–79 after first dosage. Unexpectedly, on both follow up a marked reduction in the intensity of infection with high egg reduction rates but low cure rates were recorded in the three treatment groups [84]. The investigators suggested that the lower cure rates obtained with PZQ might reflect that children treated with this drug had high infection intensities before the drug administration. It was expected that combination regimens achieved higher cure rates since drugs have antischistosomal activity and, moreover, act on different developmental stages. These results might be explained by default of assessment of viability of excreted eggs since they did not count dead eggs; thus, cure rates might underestimate the true situation. Notably, the findings contrast with those from some other reports. From earlier reports with similar treatment schedules, it was expected that higher cure rates combining antimalarial (AS, ART and MFQ) with PZQ would be obtained compared to PZQ alone [73,74,81]. The authors observed on first follow up, a conclusion whether the addition of MFQ and/or AS would expand the activity profile of PZQ targeting juvenile schistosomes could not be made [84]. Overall, PZQ monotherapy was the best tolerated treatment, which might be explained by the only one type of drug administered. In groups treated with

combination regimens, more than 90% of patients reported side effects, but it is unclear whether the adverse effects related to PZQ or systemic exposure of the antimalarials [84].

The role of MFQ in combined regimens should be investigated further in order to elucidate effects of these kind of combinations on acute schistosomiasis, and to characterize potential adverse effects of combination regimen of PZQ and MFQ.

3.2.3. Trioxalanes/Trioxaquines

Synriam™

The piperaquine phosphate was recently developed as an antimalarial drug and manufactured by Ranbaxy (Goregaon, India) as Synriam™ (SYN, Figure 3) [85]. Administration of SYN to mice infected with *S. mansoni* significantly reduced worm burdens similarly to piperaquine phosphate (up to 80%) while arterolane was less effective (31% reduction in worm burden). In addition, SYN, unlike PZQ exhibited antischistosomal activity against schistosomula and juvenile stages, indicating a potential for use in prophylaxis. Moreover, adult females are more susceptible to SYN than males that might be related to higher levels of haem inside its gut since SYN, like artemisinins, seem to be activated by this component. Moreover, administration of SYN in vivo led to general improvement in liver pathology and smaller-sized granulomas that might be a consequence of failure of eggs to produce key antigen(s) to stimulate dendritic cells/T cells [86]. It will be informative to assess the efficacy of SYN against the other human schistosomes.

PA1647

Trioxaquines (TXO) were developed against malaria and was also reported a dual mode of action: alkylation of heme by the trioxane entity and stacking of heme through aminoquinoline moiety leading to the inhibition of hemozoin in vitro [87,88]. Mice infected with *S. mansoni* were treated in day 21 p.i. with a combination PZQ and trioxaquine PA 1647 (Figure 3). In treatment with four oral doses of 25 mg/kg, the reduction of schistosomula burden was 73% while in PZQ and PA1647 monotherapy were only 24% or 18%, respectively. The results suggested an additive or synergistic effect against schistosomula. Due to this promising effect of PZQ and PA1647 against schistosomula, combinations of these drugs should be considered for clinical trials and might be relevant for use as chemoprophylaxis against schistosomiasis [89].

3.3. Other Combinations

The other combinations with nucleosides, acridine derivatives, anti-inflammatory agents, edelfosine, dipeptides, antifibrotic agents among others (Figure 4), are discussed below.

3.3.1. Nucleosides

In late 1980's, El-Kouni and colleagues [90–93] evaluated treatment of schistosomiasis by purine analogues but using the combination with nucleoside transport inhibitors. This alternative strategy emerges since unlike humans, schistosome lack de novo purine biosynthesis (required for synthesis of DNA and RNA) and dependent on the salvage pathways for purine [94]. By blocking or interfering with the parasite purine salvage pathway using purine analogues, schistosomes can be selectively deprived of vital purines. Only a few analogues were tested since they are either not efficiently metabolized to nucleotide level by parasite or are also toxic to mammals. Mice infected with *S. mansoni* were injected with combined regimen of nitrobenzylthioinosine 5′-monophosphate (NBMPR-P, 25 mg/kg per day for 4 days) and high doses of tubercidin (5 mg/kg per day for 4 days). Notably, these doses were highly toxic to parasite but not to the mice. The treatment resulted in an impressive decrease in the number and copulation of worms, which consequently decreased the number of eggs in the liver and intestine. All eggs found were dead. Histopathological examination of livers showed lesions with dead worms and regeneration of normal tissue around old granulomas [89]. Similar results have been achieved with this combined therapy against *S. japonicum* [91]. This

combination was also assessed on advanced stages of schistosomiasis by administration drug combination on 5, 6, 7 and 8 weeks p.i. in heavily infected mice. In the long-term progress of the disease (22 weeks p.i.), the efficacy of combination therapy was monitored. In this case, the reduction of number of worms and eggs in liver and small intestine were less evident [91]. Additionally, the combined therapy was effective in preventing the formation of novel egg granuloma although activity was not seen against existing granuloma. In another study, the therapeutic efficacy of NBMPR-P in combination in mentioned dose with potential antischistosomal purine analogues, nebularine (37 mg/kg/day), 9-deazaadenosine (1 mg/kg/day), toyocamycin (1.6, 1.8, 2.0 and 2.2 mg/kg/day for 4 days) and dilazep (25 mg/kg/day) was tested in *S. mansoni* infected mice. Administration of NBMPR-P combined with 9-deazaadenosine did not affect the parasites. However, combinations of NBMPR-P or dialazep with tubercidin or nebularine were highly toxic to schistosomes, achieving similar results as described above [93,94].

Despite these encouraging results in the laboratory, human trials with these drugs have not been reported, likely reflected potential toxicity risks associated to with these nucleosides.

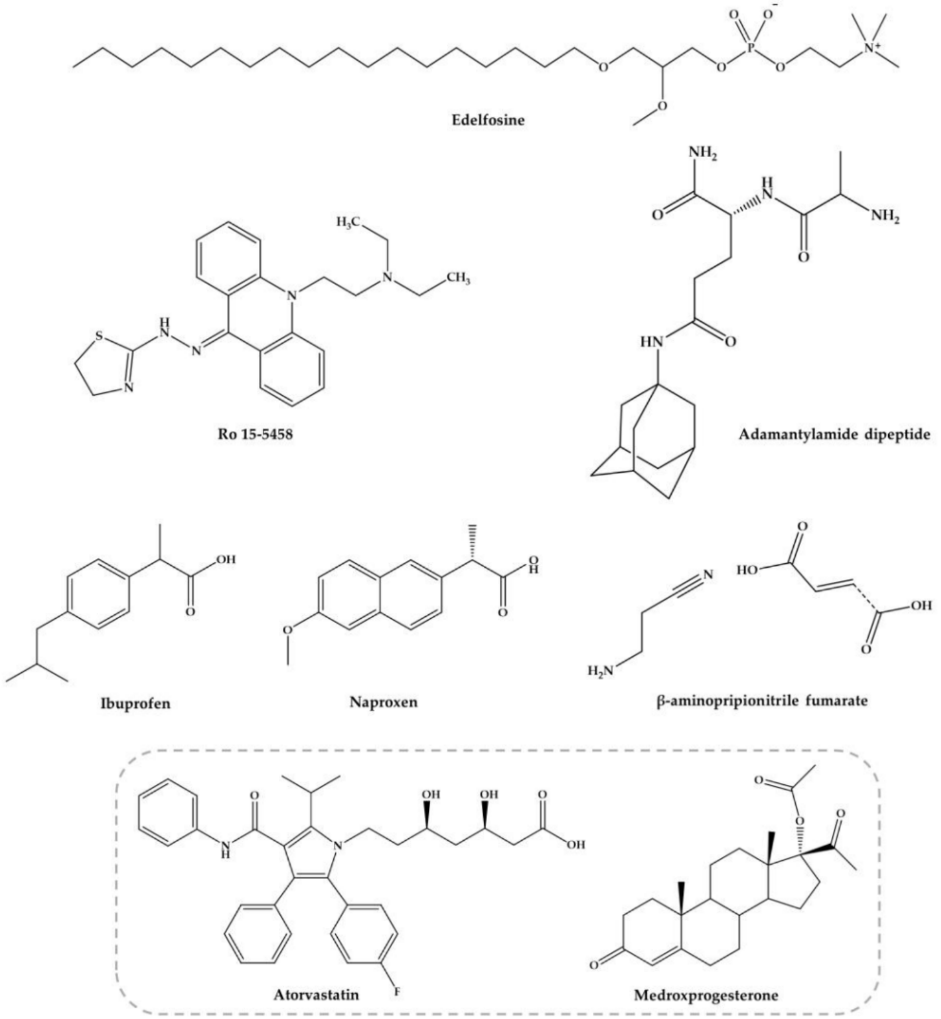


Figure 4. Several compounds within different properties against experimental *S. mansoni* infection. Compounds in the grey square were studied in combination, and others were studied alone and combined with PZQ.

3.3.2. Acridine Derivatives

Ro 15-5458 (Figure 4) is an acridine derivative from class 9-acridanone-hydrazones that have been developed by Hoffman-La Roche (Basel, Switzerland) [95]. The possible synergistic and/or

additive effect of PZQ with Ro 15-5458 was evaluated against two different strains of *S. mansoni* in mice, i.e., CD-susceptible and SO_4-resistant strains. The treatment with a single curative dose of PZQ or Ro 15-5458 were also compared to those achieved by drugs in combination at doses corresponding to one-third of the curative dose of PZQ and Ro 15-5458. Combination of PZQ and Ro 15-5458 demonstrated to be beneficial as regard the percentage of parasite reduction and hepatic worm shift (99.4% to 100%, respectively in the CD-susceptible mouse strains, compared to 84.1% and 34.8% in the SO_4 resistant strains) [95]. Moreover, it was observed a decrease in the number and size of granulomata with disappearance of pathological changes in hepatocytes. The authors considered that Ro 15-5458 has excellent antischistosomal properties and should be considered candidate for drug discovery and development pipeline [95,96]. However, thus far, no further investigations in order to evaluate mutagenicity and carcinogenicity of this compound as basis for possible clinical trials with humans.

3.3.3. Anti-Inflammatory Agents

The nonsteroidal anti-inflammatory drugs (NSAIDs) were found to suppress the inflammatory process delaying hypersensitivity reaction in schistosomal hepatic granulomas and fibrosis [97]. Ibuprofen and naproxen (Figure 4) alone did not reduce significantly the worm distribution, egg load or change the oogram pattern when compared with the infected control [98]. Nevertheless, when administered in combination ibuprofen and naproxen with PZQ caused a slightly increase of percentage of dead ova (96.1% and 97.3%, respectively) at 16 weeks p.i. with a marked reduction of mature ova while PZQ alone reduce the number of worms to 95.6% [98]. These evidences demonstrated that they do not possess any antischistosomal activity, although they play a role in amelioration of biochemical and histopathological consequences related to intensity of infection [98]. Administration of these NSAIDs alone (200 mg/kg for two weeks) significantly reduced the granuloma diameter while has no effect on their type nor in serum levels of hepatic enzymes and circulating antigen. Treatment with ibuprofen and naproxen combined with PZQ (2 × 500 mg/kg) improved the parameters mentioned resulting in marked reduction in the mean granuloma diameter and circulating antigen which was more pronounced with naproxen than ibuprofen. These evidences suggest a combined action of PZQ in elimination of parasite and anti-inflammatory properties of ibuprofen and naproxen. Authors considered that treatment with NSAIDs is not preferable without PZQ but may be used as adjuvant in treatment of pathologies associated to infection [98].

3.3.4. Edelfosine

Yepes and co-workers [99] study antischistosomal activity in vitro of a synthetic lipid compound, edelfosine (EDLF) (Figure 4), against schistosomula and its combination with PZQ in vivo. It has been reported that EDLF display anti-inflammatory properties [96] and modulate cytokine production such as interferon-γ (INF-γ), interleukin-2 (IL-2) and interleukin-10 (IL-10) [100,101]. This modulation might be relevant since cytokine production by host blood cells after stimulation with parasite antigen reflects a dominant T helper 1 (Th1) response during acute phase, producing interferon-γ (IFN-γ) and IL-2. Following parasites maturate, mate and produce eggs was followed by a developing egg antigen-induced regulatory T cell and T helper 2 (Th2) response, that downregulates the production and effect functions of the pro-inflammatory Th1 mediators accompanied by granuloma formation [102,103].

In a preliminary experimentally studies EDLF induce interruption of oviposition in vitro as well as significant reduction in worm burden in vivo being most effective against male worms [104]. Contrary to PZQ, EDLF is active against schistosomula of *S. mansoni*. In addition, authors study the effects of the combination of PZQ (100 mg/kg/day) plus EDLF (45 mg/kg/day) administered in *S. mansoni* infected mice daily 3 days prior to infection until eight days p.i. Combine regimen not only acts on parasite through the elimination of developmental stages; as well on histopathological parameters inducing reduction of hepatomegaly, granuloma size and immunological effects downregulation of Th1, Th2 and Th17 responses reflecting in inhibition granuloma development and up and down-regulation of IL-10 on early and late post-infection times, respectively. Consequently,

this regulation potentiates anti-inflammatory actions and favoring resistance to re-infection. In addition, reduction in the number of blood granulocytes in late post-infection in comparison to infected untreated animals [99]. Taken these together it has been suggested that this combine regimen treatment may provide a promising and effective strategy for a prophylactic treatment of schistosomiasis.

3.3.5. Antifibrotic Agent, β-Aminopropionitrile

The combination with non-schistosomal drugs such as β-aminopropionitrile-monofumarate salt (Figure 4) with PZQ was evaluated by Egyptian researchers in mice infected with *S. mansoni* [105]. The findings reveal that the combined regimen reduced total worm burden reduction (100%) and markedly reduced the egg load in the liver and intestines. Moreover, combination regimen revealed the highest score of resistance to reinfection, compared to the other groups given each drug alone [105]. Similar results were achieved with combine regimen of PZQ and β-aminopropionitrile (BAPN) in *S. mansoni* infected mice [106]. Modulation of granuloma formation by combined antifibrotic/PZQ therapy significantly alters the process of egg granuloma formation and alleviates the host resistance to challenge infection. Treatment results in relatively small sizes granuloma in comparison to large and irregular form of granulomas detected in intestinal tissues on control mice. However, the mechanism by BAPN reduces the number of liver granulomas is still unclear. In addition, treated mice with combine regimen showed decreased liver and spleen weights and a significant reduction in the number of eggs trapped in both liver (86%) and the intestine (99.1%), in comparison to untreated mice and those given PZQ alone [106]. According to authors, these results suggested that administration of PZQ combine with BAPN might also be useful as adjuvant in amelioration of pathologies associated to infection.

3.3.6. Adamatylamide Dipeptide

Botros et al. [107] investigate the possible use of adamantylamide dipeptide (AdDP) (Figure 4) as adjuvant therapy to PZQ in mice infected with PZQ-insusceptible and susceptible *S. mansoni* isolate in a trial to increase the susceptibility of this isolate to the drug. Seven weeks p.i., the experimental group received AdDP (5 mg/kg) in addition to PZQ in reduced dose (3 × 100 mg/kg) and groups received PZQ and AdDP alone. In mice infected with PZQ-susceptible and insusceptible *S. mansoni* isolates, intraperitoneal injection of AdDP alone did not significantly reduce the total number of worms suggesting that dipeptide did not present antischistosomal activity. Treatment with AdDP and PZQ in reduced dose resulted in significantly higher antischistosomal efficacy than PZQ in reduced dose, demonstrating that AdDP reduced the effective dose of PZQ. This efficacy obtained together with granuloma diameter reduction and diminution in percentage of fibrotic areas was also comparable to that observed in mice treated with full dose of PZQ. The results might be related to synergistic effect of PZQ and AdDP when administered in combine regimes; in fact, AdDP enhance antischistosomal activity and ameliorate the hepatic inflammatory reactions [107].

3.3.7. Atorvastatin and Medroxyprogesterone Acetate

Soliman and Ibrahim [108] conducted a study in order to evaluate the influence of long-term administration of lipid lowering agent atorvastatin (AV, Figure 4) combined with injectable contraceptive medroxyprogesterone acetate (MPA, Figure 4) on tegumental structure and survival of *S. haematobium* worms. MPA was administered intramuscularly (0.1 mg/kg) at days 7 and 35 p.i. followed by AV treatment regimen (0.9 mg/kg for 49 consecutive days) in *S. haematobium*-infected hamsters. Long-term administration of AV induced mild to severe morphological alterations, particularly in the tegument of schistosomes. Similarly, treatment with AV concurrently with MPA significantly increased tegumental damage and significantly reduce the total numbers of *S. haematobium* worms recovered from hamster infected (51.3%). No significant difference was found in both combine regimen and AV monotherapy (46.2%). Female worms were less susceptible to both drug regimens compared to males [108]. The investigators correlate significant reduction of the

recovered worms as result of both treatments with tegumental damages induced, in addition to the possible influence of AV on biochemical pathway of the parasite [108]. Furthermore, tissue egg load and oogram pattern decreased in hamsters treated with the combination regimen which might be related not only to reduction of recovered worms but also with suppression of egg production due to inhibitory influence of AV on the enzyme 3-hydroxy-3-methoxyglutaryl-coenzyme A (HMG-CoA) reductase, which is critical for regulation of egg production by the parasite. Inhibition of HMG-CoA should be investigated since it might be considered as a potential drug target [108]. Studying schistosomal and mouse liver HMG-CoA reductase activity was also observed elevated quantity in the liver but very reduced in the parasite [109]. In other work, the parasite death by statins or specific RNAi of HMG-CoA is associated with activation of apoptotic caspase activity [110].

4. Antioxidants: A New Chemotherapy against Schistosomiasis?

In recent times, studies evaluating efficacy of antioxidants, alone or concurrently with antischistosomal drugs targeting not only parasite but also pathologies associated to infection have been reported [111–140] (Table 1 and Figure 5).

Most antioxidants assessed have shown potential schistosomicidal activity both in vitro and in vivo, against mature [111,113,116,124,127,129,130,132,134,135] as well as immature [112,118,130,131] *S. mansoni* and *S. japonicum* [120] developmental stages, counteracting one of major drawback of PZQ. Even though the exact mechanism of action remains unclear, some antioxidants including pholoroglucinol derivatives and extracts of *B. trimera* affect motor activity of the worms in vitro. This phenotype is an insightful indicator of schistosomicidal activity, since it reveals perturbation/dysfunction of elements of the neuromuscular system [117]. In addition to movement, schistosomes use their neuromuscular systems to control the muscles of the oral and ventral suckers, which allow the worm attach to the host, musculature supporting internal organs including the reproductive excretory and digest tract, and maintenance of the female within the gynecophoral canal of the male [141,142].

Additionally, antioxidants such as limonin, phloroglucinol derivatives (Figure 6), and extracts of *B. trimera* and *A. sativum* induce severe tegumental alterations [112,113,117,127] which is noteworthy since the tegument plays a crucial role in host-parasite interactions, nutrient uptake for parasite growth and development, and protection against host responses [143]. Furthermore, several antioxidants have impaired worm coupling [112,130,132,137], a fundamental process for schistosome viability inside the host human and for establishing the infection. During pairing, the female is maintained in the gynecophoric canal in the male body for sexual maturation and egg production to occur.

The induction of separation of males and females by antioxidants reduce or even cease the release or production of the eggs [111,117,120,123,127–129], which are the major cause for the formation of inflammatory granuloma on target organs, and transmission of schistosomiasis [144]. In fact, the pathology associated with schistosomiasis is largely attributed to the intense of granulomatous inflammation and subsequent fibrosis induced by parasite eggs trapped in host organs such as liver, intestine and bladder. The toxic products released from egg destroys the host tissue cells and the antigenic material stimulates the development of larger inflammatory reactions leading to formation of granulomas around eggs [145]. As presumed, in the presence of parasite, the host immune system reacts in a manner that involves reactive oxygen species (ROS) leading to increase of oxidative process during the course of infection [145]. For example, eosinophils, one of the components of *Schistosoma*-induced hepatic granulomas, generate hydroxyl radical (OH) and the super oxide anion (O_2) [146,147]. Several host organs, especially the targets, are affected by increased eosinophil peroxidase activity and imbalance in the antioxidant defense mechanism causing these organs to be shifted to a pro-oxidant state [145]. The ultimate aim of ROS generation may be killing the parasite eggs; yet, they alter liver homeostasis decreasing antioxidant defenses and increasing the liver enzymes such alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) that are measures of liver affection [145].

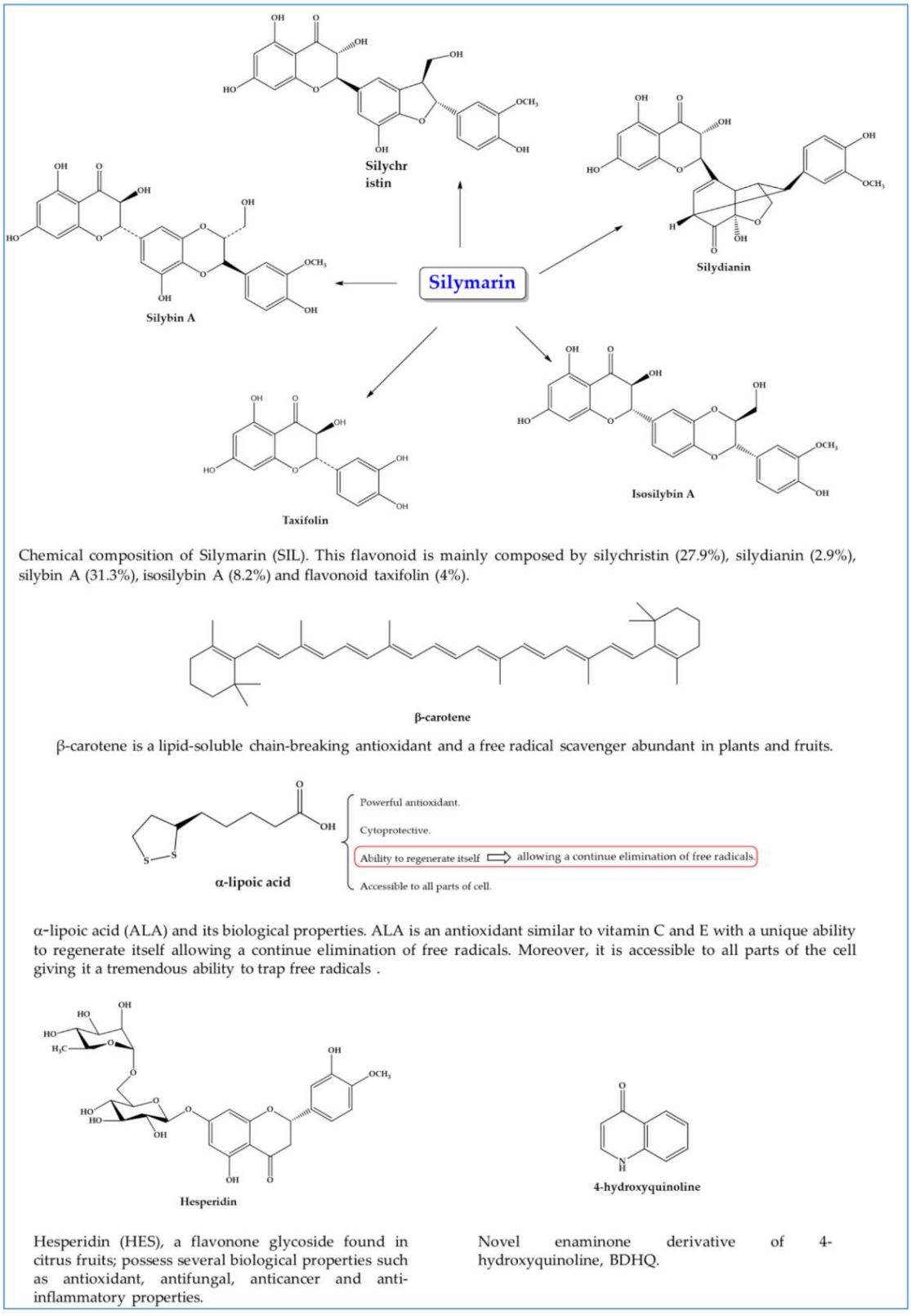


Figure 5. Chemical structures of compounds with antioxidant properties against schistosomiasis.

Table 1. Reports of antioxidant effects on developmental stages of schistosomes and infection in murine models.

Compound	Aim/Study	Treatment	Findings/Outcomes	Ref.
M. armillaris	Antischistosomal and antioxidant activity of essential oil on normal and infected *S. mansoni* mice in comparison to PZQ.	Oil 150 mg/kg orally from second week p.i., twice week for 6 weeks; PZQ 600 mg/kg, orally for 2 consecutive days, 8 weeks p.i.	Administration of PZQ and *M. armillaris* ameliorate the levels of GSH and lipid peroxides (MDA); Restored the activities of SOD and catalase; *M. armillaris* enhance antioxidant defense system reducing disease complications.	[111]
Limonin	Antischistosomal activity in vitro and in vivo harboring juvenile and adult *S. mansoni* worms.	Oral administration in a single dose of 50 or 100 mg/kg on day 21 p.i.; Same dose given on 56 p.i.	Limonin is more effective against immature stages rather adult worms also induce tegument alterations; Reduction of worm burden: at day 21 p.i. 70.0% and 83.3%; and day 56 p.i. 41.09 and 60.27%. Significant reduction in the hepatic (34.90% and 47.16%) and intestinal (46.67% and 56.1%) tissue egg load associated the oogram pattern with elevated dead egg levels; Also, ameliorate hepatic pathology with reduction in size and numbers of granulomas.	[112]
Pholoro-glucinol derivatives	Evaluation in vitro schistosomicidal activity of aspidin (AS), flavaspidic acid (FAA), methylene-bis-aspidinol (MbA) and desaspidin (DA) against *S. mansoni* adult worms.	AP-25 to 100 μM FAA-50 and 100 μM MbA-100 μM DA-25 to 100 μM	AP and FAA decrease motor activity with tegumental alterations while MBA and DA also decrease motor activity but without tegumental alterations. At highest concentrations viability of worms were similar to positive controls (PZQ); Egg production and the development of eggs produced were inhibited; Probably, in vitro activity is related to the inhibition of oxidative phosphorylation pathways.	[113]
Hesperidin	Evaluation of antischistosomal activity in vitro and in vivo and compared to PZQ. Effect on parasite antigens. Treatments were administered on 6th week p.i.	In vitro: 50, 100 and 200 μg/mL. In vivo: Hesp-600 mg/kg bw (6 injections, 2 injections per week for 3 consecutive week); PZQ (2 consecutive days with 500 mg/kg bw.	In vitro: At highest concentration, all males and females were dead while lower concentration had moderate effect. No activity on oogram pattern was seen. In vivo: Reduction of numbers of males, females and possibly worm pairs and total worm burden counts (47.5%) but was not higher than PZQ; significantly reduced tissue egg load. Augmented the mouse IgG response against soluble worm antigen protein, soluble egg antigen and cercarial preparation of *S. mansoni*.	[114]
α-Lipoic acid	Study combined effect of ALA with PZQ on liver fibrosis induced by *S. mansoni* infection in mice.	PZQ-500 mg/kg divided into 2 doses 9 weeks p.i.: PZQ (same described) + ALA (single dose 30 mg/kg) daily for two months.	Combine regimen results in reduction in the worm burden (ALA: 7.63 ± 1.49; PZQ: 6.13 ± 1.89; PZQ + ALA: 36.50 ± 10.80), egg count and granuloma size. Recovered the level serum of ALT, AST and GGT and increased the tissue level of GSH and decreased MDA (biomarkers of antioxidant function and stress oxidative, respectively).	[115]
Resveratrol	Investigate effect of Resv on oxidative stress imposed on liver, lung, kidney, brain and spleen of *S. mansoni*-infected mice.	20 mg/kg once daily for 2 weeks	Improvement of lipid metabolism and antioxidant profile by Resv which were not only restricted to liver but also other vital organs. Specific biomarkers of lung and brain homeostasis also showed remarkable improvement.	[116]
B. trimera	Assessment of antischistosomal activity agaisnt *S. mansoni* adult worms in vitro.	4 concentrations of 24, 48, 91 and 130 μg/mL.	Antischistosomal activity at highest concentrations with significant reductions in motility; Total inhibition in egg laying when parasites were exposure to sub-lethal concentrations and separations of all couples.	[117]

			Morphological changes on the tegument of worm's males and females.	
	In vitro and in vivo efficacy of aqueous fraction and dichloromethane extracts against schistosomula, juvenile and adult worms of *S. mansoni*.	In vitro: Same as above In vivo: Single doses 40 and 200 mg/kg of *B. trimera* and PZQ 4 after 3 and 30 days p.i. and 60 p.i.	In vitro: Similar results described. In vivo: *B. trimera* exhibits major schistosomicidal effects in vivo against immature and adult worms (significantly female worm, 68–75%, reduction and number of eggs/g in faeces); Significant reduction in relation to number and size of granulomas.	[118]
Melatonin	Assessment protection against oxidative stress induced by schistosomiasis mansoni.	3.55 mg/kg daily for 30 consecutive days starting from first day p.i.	Decreased in total leukocyte count: Markedly reduced the fibrotic areas, small diameter of granuloma with few collagen fiber depositions; ameliorate liver architecture and glycogen content.	[119]
	Establish an immunization program using *S. mansoni* adult worm antigen and cercarial antigen alone or concurrently with Mel in attempt to enhance efficacy against infection in mice.	30 μg/mL CAP or SWAP on first day and 20 μg/mL on 4th day p.i.; On 7th day all hamsters were infected. Mel same regimen as above.	Mel alone did not result decrease of worm burden reductions (CAP: 538%; CAP + Mel: 67.01%; SWAP: 56.4% and SWAP + Mel: 99.3%). Highly significant reductions in egg load in liver and alteration of oogram pattern: high percentage of immature eggs and few dead eggs. Improved the oxidative status in the immunized groups. No antibody response was observed in the groups immunized with SWAP + Mel while low antibody level was observed in CAP + Mel.	[120]
	Investigate oxidative processes in mice infected with *S. mansoni*	10 mg/kg, 2 weeks after *S. mansoni* infection until end of experiment; or daily for 30 days	Mel did not restore glutathione levels (although were tendencies for that); Increase SOD activity (but not statistically significant); Reduction of AST levels; Reduction of granuloma formation and highly protective against pathological changes not only in liver but kidney; Mel has multiple direct and indirect antioxidant actions and its ability to stimulate antioxidative enzymes and mitochondrial oxidative phosphorylation.	[121]
4-Hydroxy-quinolin-2(1H)-one (BDHQ)	Evaluation potential activity on murine schistosomiasis. For that mice were sacrificed on different weeks p.i.: 3 (for schistosomula) and 6 (for adult worms)	BDHQ: Lower dose—10 mg/mL for consecutive days; Higher dose on same regimen; PZQ: 2 times of 500 mg/kg 2 consecutive days on different weeks.	Antischistosomal activity against immature and mature worms; Destructive effects on the female and male genital systems; Antischistosomal activity may be due to its mixed cellular and humoral immunologic mechanisms, as demonstrated by the significant increase of serum levels of IgE and IFN-γ.	[122]
4-Hydroxy-quinolin-2(1H)-one (BDHQ)	Evaluation of antioxidant and antigenotoxic effects alone or combined with PZQ.	PZQ, 0 or 500 mg/kg BDHQ, 600 mg/kg PZQ (250 mg/kg) + BDHQ (300 mg/kg) for 2 consecutive days	BDHQ alone or combined resulted in highly significant reduction in total worm burden (7 weeks p.i. PZQ: 86.37%, BDHQ: 79.22%; PZQ + BDHQ: 91.84%; 9 weeks PZQ: 94.72%, BDHQ: 92.32%; PZQ + BDHQ: 95.54%), associated with significant reduction in the hepatic tissue egg load; Drugs alone reduced the granuloma size and inflammatory cells. These parameters were improved with combine regimen; Significant decrease in MDA level accompanied with highly increase in NOx level with combine regimen, in addition to increase in the activities of both SOD and CAT; Remarkable significant decrease in % DNA fragmentation reaching a level close to control; These suggest a synergistic action attributed to different mechanism of action of both drugs that achieved the same or higher	[123]

			levels of efficacy using smaller doses of either agent.	
	Assessment of parasitological and biochemical parameters on *S. mansoni* infection in mice.	10, 20 or 25 doses of 10 mg/kg Syl suspended on carboxymethyl-cellulose at 55 days p.i.	Did not show antischistosomal activity; Reduced granulomatous and hepatic fibrosis. At acute schistosomiasis may result in a mild course of murine schistosomiasis and minimize the deleterious effects.	[124]
Sylimarin	Anti-inflammatory/antifibrotic effect alone and combined with PZQ.	Syl: —4th week p.i. (3 weeks before PZQ therapy) —12th week p.i. (5 weeks after PZQ); PZQ (7th week p.i.) Syl + PZQ	Syl alone: Partial decrease of worm burden (26.55 and 39.39%) and decrease hepatic tissue egg load with an increase in percentage of dead ova; Modulation of granuloma size and conservation of hepatic GSH. PZQ: Complete eradication of worm, egg and alleviated liver inflammation and fibrosis; Combine regime: Improvement of liver function and histopathology whether acute and chronic infection may due to a combine action of anti-inflammatory, anti-fibrotic actions, in addition to the antioxidant properties of silymarin. Syl did not interfere or affect the antischistosomal activity of PZQ. Worm burden reduction 97–100%.	[125]
A. sativum	Antischistosomal activity against *S. japonicum* cercariae in vitro and in vivo.	In vitro: 10^{-2} to 10^{-6} (*v*/*v*) concentration. In vivo: Pre-treated with garlic, then mice were infected.	Garlic oil displays marked activity agaisnts *S. japonicum* cercariae and may be used as agent to prevent *S. japonicum* (pre-exposure garlic oil at 10^{-4} and high showed total inhibition of infection).	[126]
A. sativum	Assess potency and the immunomodulatory response in enhancing the host immune system caused by *S. mansoni* in mice at different stages of worm.	100 mg/kg body weight from 1 to 7 days p.i., 14 to 21 or 1 to 42 days p.i.	Morphologic alterations in the parasite tegument; significant decrease in worm burden, hepatic and inestinal ova count. Decline in granuloma number and diameter; Reduction in serum TNF-α, ICAM-1, IgG and IgM after 7 and 42 days p.i.; garlic oil enhance host immune system.	[127]
	Ability of both oils to offser infectivity as well as metabolic disturbances induced by *S. mansoni* infection	5 mL/kg body daily separately for 8 weeks on healthy control and infected groups. On infected groups oil were given 24 h p.i.	Reduced worm burden (garlic: 67.56% and onion: 75.97%) and ova count; normalized liver functions enzymes; effect may be induced by improving the immunological host immune system and their antioxidant activities.	[128]
A. sativum + *A. cepa*	Effect of both oils alone and mixed or concurrently with PZQ on biochemical parameters of experimentally infected *S.mansoni* mice.	*A. sativum* or *A. Cepa*, 2 g/100 g body weight daily for 45 consecutive days. PZQ: 500 mg/kg bw on 2 successive days 45 days p.i.	Significant reduction in worm burden (PZQ: 95.8%; onion: 66.29%; PZQ + onion: 99.1%; garlic: 73.41; garlic + PZQ: 99.3%; garlic + onion: 74.63; garlic + onion + PZQ: 99.7%); Reduction hepatic and intestinal eggs and oogram count; Suppression in granuloma tissue formation and diminutive histopathological changes; Improvement of liver architecture and attenuated the decrease of tissue antioxidant enzymes	[129]
	Antischistosomal activity in vitro against *S. mansoni* miracidia, cercariae, schistosomula and adult worm. Effect in vivo on lipid peroxidase and antioxidant enzymes.	In vitro: 0.5–5 ppm In vivo: Same described above.	Lethal effect of both antioxidant against all developmental stages; Inhibition of coupling; Powerful reducing capacity demonstrated in DPHH radical scavenging and NO; Both plants enhance host antioxidant system indicated by lowering in lipid peroxide and stimulation of SOD, CAT, GR, TrxR and SDH enzyme levels which could turn render parasite vulnerable.	[130]
	Antischistosomal activity against	In vitro: Serial concentrations (0.5–5	Antischistosomal activity against miracida and cercariae; Separation of coupled worms;	[131]

	miracidia, cercariae and adult worms in vitro. Effect on some antioxidants enzymes.	ppm) for miracidia and cercariae. Adult worms, 10–110 ppm.	Inhibition of egg laying by adult female worms; Significant inhibition of parasitic antioxidant enzymes (SOD, GR and GPX) and enzymes glucose metabolism (HK and G-6-PDH), higher in males than females.	
Nigella sativa	Effect in protection against oxidative stress in experimentally infected mice with *S. mansoni*.	*N. sativa* oil (1.14 g/kg orally) for 30 consecutive days from first day p.i.	No suppressive effect on granuloma formation in intestine; Did not improve the liver architecture; Noticeable degree of protection represented in less severe pathological changes, particularly the frequency of inflammatory reactions.	[126]
	Study effect the oil on liver functions and antioxidant ability on experimentally infected mice with *S. mansoni*.	2.5 and 5 mL/kg orally either alone or in combination with PZQ (500 mg/kg for 2 consecutive days)	*N. sativa* alone: Reduce the number of *S. mansoni* worms in the liver; Total worm burden: 22% e 32%, respectively, while PZQ: 98%; Decreased total number of ova deposited; Increased the number of dead ova; Reduced the granuloma markedly; Partially correct alterations in serum levels of ALT, GGT, activity as well as the Ab content. Failed in the liver restore either LPD and GSH content or LDH (lactate dehydrogenase) and SOD activity to normal level. *N. sativa* + PZQ: Improved most parameters with most prominent effect was further lowering in dead ova number over that produced by PZQ. Total worm burden: 98% and 99%.	[132]
Nigella sativa	Investigate immune mechanism possibly involved in the amelioration of histopathological changes in liver of *S. mansoni* infected mice treated alone or in combination with ART or PZQ.	*N. sativa*: orally with 0.2 mg/kg of body weight for 4 weeks starting from 1st day p.i. ART: intramuscularly single dose of 300 mg/kg of body weight after 49 days p.i. PZQ: 500 mg/kg for 2 consecutive days	*N. sativa* as well as the combination of ART or PZQ resulted in significant increase in IL-2, IL-12 and TNF-α activities in *S. mansoni* infected mice as well as treatment of NS in non-infected. *N. sativa* in combination with ART or PZQ accelerate healing pathological granulomatous lesions of liver architecture and improved host immunity by stimulating cytokines.	[133]
	Antischistosomal activity and antioxidant effects of NS alone or combined with garlic extracts on experimentally *S. mansoni* infected mice.	Garlic extract 125 mg/kg p.i. and NS oil 0.2 mg/kg alone or combine for successive 28 days, starting 1st day p.i.	All treatment regimens significantly affected oogram pattern: treatment with compounds alone resulted in reduction of percentage of mature eggs while combine regimen resulted in increase of percentage of dead eggs. Administration of garlic extract prevent GSH depletion on infected mice. Combine regimen had more significant effect on serum enzymes (AST and ALP).	[134]
Curcumin	Assess curative effect of oil extract in liver cells of *S. mansoni* infected mice in compaison to PZQ	PZQ: 500 mg/kg by 2 consecutive days Extract: 300 mg/kg bw after one month p.i., twice a week for 2 months	Curcumin normalize the concentration of protein, glucose, AMP-deaminase and adenosine deaminase which were altered by infection Lowered pyruvate kinase level while PZQ induce more elevation; More potent rather PZQ in reducing egg count but no lowering worm burden. Most likely, antifecundity effect of curcumin might be involve in impairment or adult worms.	[135]
	Evaluation of schistosomicidal activity in vivo and immunomodulation of granulomatous inflammation and liver pathology in acute *S. mansoni* infection.	Total dose 400 mg/kg bw divided into 16 injections (2 injections per week for 8 consecutive weeks) starting from the first week of infection.	Effective in reducing worm (44.4%) and tissue-egg burdens; Reduction granuloma volume and liver collagen (79%); Restore hepatic enzymes activities to normal levels and enhanced catalase activity; Low serum level of both IL-12 and TNF-α; Augmented specific IgG and IgG1 responses against both SWAP and SEA.; It modulates cellular and humoral responses.	[136]

	Evaluation its role on induction of apoptosis and oxidative stress in couples of adult *S. mansoni* worms in vitro	1.56 to 100 μM incubated for 6, 12 or 24 h.	Significantly decreases the viability of adult female and male worms; Induce separation of couples and morphological alteration on mitochondria; Induce formation of SOD and increase its activity in adult worms; Alters several oxidative stress parameters in adult worms such decrease of GST, GR and GPX culminating in the oxidation of protein: Generates oxidative stress followed by an apoptotic-like event in adult worms, which ultimate leads to their dead.	[137]
β-carotene	Evaluation the protective effect on experimentally *S. mansoni* infected mice and on major enzymes activities involved in liver redox.	PZQ, 7 weeks p.i., 500 mg/kg (full dose) or PZQ ED_{50} 74.64 mg/kg βC, 2.7 mg/kg, 1 week before infection. βC + PZQ ED_{50} as mentioned	Produced significant reduction in worm burden (total number of worms: PZQ: 11.57 ± 0.59; PZQ (full dose): 0.46 ± 0.14; βC: 17.64 ± 1.11; βC + PZQ: 8.38 ± 0.51) accompanied with increase of dead ova and decrease in percentage of mature ova; reduced liver granuloma diameter. Combined regimen improved these parameters. Combined regimen improved the effect of antioxidant enzymes (as GPX and GST) and increase serum ALT and GGT. βC has protective effects against liver fibrosis which may be due to ability to encounter or minimize the formation of schistosomal products.	[138]
N-Acetyl-cysteine	Study immunopathological changes in murine schistosomiasis alone or in combination with PZQ.	NAC (200 mg/kg/day on 1st day after infection for acute phase; On 45th for the intermediate; 59 and 75th for chronic phases. PZQ (100 mg/kg) from 45th to 49th day p.i.	NAC alone did not present any schistosomicidal activity; animals treated with NAC and/or PZQ showed a reduction in the size of granulomas and those treated with NAC exhibited a lower degree of fibrosis. NAC functions as a direct scavenger of NO and peroxinitrite which are related to reductions of IFN-γ levels and increasing of IL-10 synthesis; Induce an immunomodulatory effect and reduce liver damage during granulomatous inflammation.	[139]
	Investigate ability of NAC to enhance potential of ART against adult *S. mansoni* worms and evaluates protective role on oxidative stress.	NAC-300 mg/kg 5 days a week for 4 weeks ART-300 mg/kg 7 weeks p.i. NAC + ART (as described)	Combine regimen approximately recovered levels of serum enzymes, content of GSH and activities. Decrease the total number of worms and hepatic ova count. ART alone produce valuable modulations in the hepatic activities; NAC may prevent experimental liver injury by modulating and enhancing GSH content and GSH-dependent antioxidant enzyme activities. Total worms: ART: 7.6 ± 1.5; NAC: 17.7 ± 1.5; NAC + ART: 3.3 ± 1.1.	[140]

PZQ: praziquantel; Resv-Resveratrol; Mel-melatonin; BDHQ: 4-hydroxy-quinolin-2(1H)-one; ART: artemether; NS: *Nigella sativa*; βC: β-carotene; NAC: *N*-acetyl-cysteine; AS: aspidin; FAA: flavaspidic acid; MbA: methylene-*bis*-aspidinol; DA: desaspidin; Syl: sylimarin; p.i.: post-infection bw: body weight; GSH: glutathione; GR: glutathione reductase; SOD: superoxide dismutase; GGT: gamma-glutamyl transferase; CAT: catalase; TrxR: thioredoxin reductase; SDH: succinate dehydrogenase; GPx: glutathione peroxidase; HK: hexokinase; G-6-PDH: glucose-6-phosphate dehydrogenase; DPHH: 1,1-diphenyl-2-picrylhydrazyl; NOx: nitrogen oxide; IgG: immunoglobin G; IgE: immunoglobin E; IgM: immunoglobin M; IL-2: interleukin-2; IL-10: interleukin-10; IL-12: interleukin-12; IFN-γ: interferon gamma; TNF-α: tumor necrosis factor α; ICAM-1: intracellular adhesion molecule; ALA: alanine aminotransferase; AST: aspartate transaminase; MDA: malondialdehyde; LPD: lipoamide dehydrogenase; LDH: lactate dehydrogenase; ALP: alkaline phosphatase; AMP: adenosine monophosphate; SEA: soluble egg antigen; CAP: cercarial antigen preparation; SWAP: soluble worm antigen preparation.

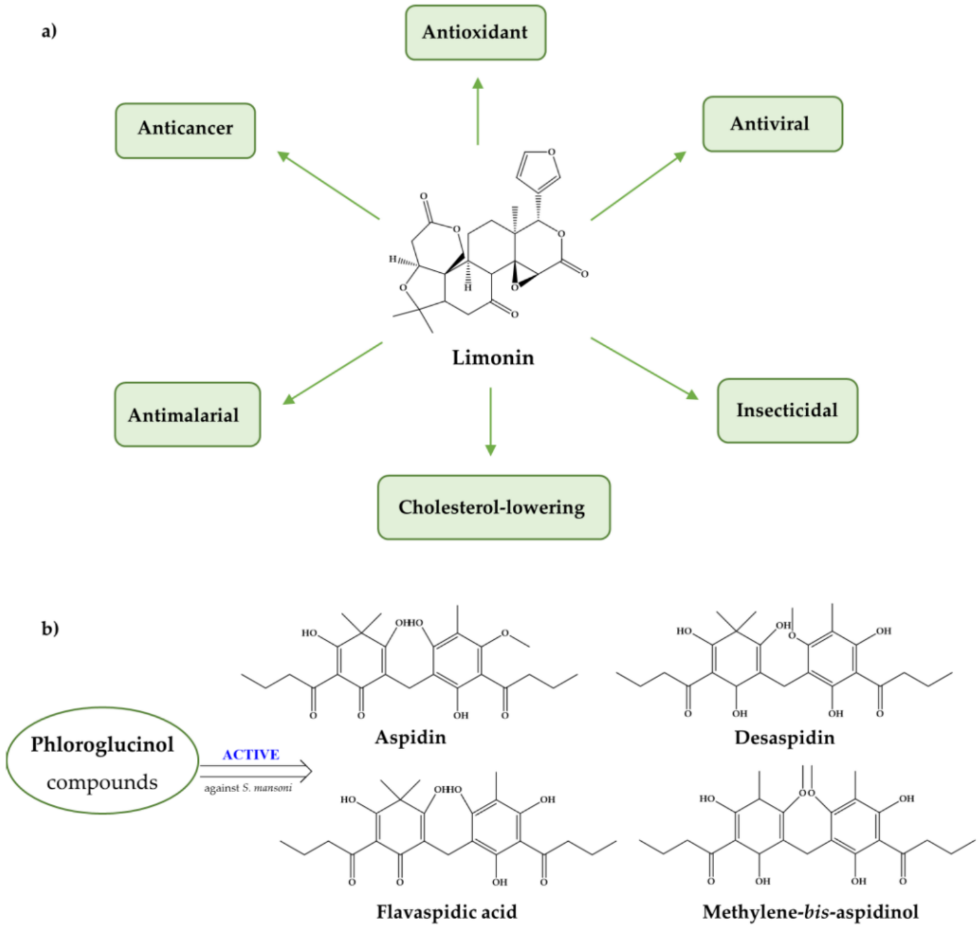


Figure 6. (**a**) Phytochemical limonin is abundant in citrus fruit and displays several pharmacological activities; (**b**) Phloroglucinol compounds, aspidin (APD), flavaspidin acid (FPA), methylene-*bis*-aspidinol (MbA), desaspidin (DSP), desaspidinol (DSPL) with activity against adult *S. mansoni* worms, they impede motor activity of the parasite [112].

Mammalian cells has adopted a chain of antioxidant system, either enzymatic or not, to limit the overcome the harmful imposed by ROS. Enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are key players in defense against ROS. The SOD hastens the speed of dismutation of superoxide to hydrogen peroxide (H_2O_2). Afterwards, comes the action of catalase which transform H_2O_2 into H_2O and O_2 and tackle the chains of unsaturated fatty acids present in membranes and other macromolecules such as proteins. In cell, reduced glutathione (GSH) play a role in many biological processes, including the synthesis of proteins, maintenance of cellular activity, xenobiotics and reactive aldehydes detoxification (such as malonaldehyde, MDA), metabolism and cell acting protections against free radicals [145]. Some studies have suggested that a direct link to parasite load to intensity of inflammatory reaction and antioxidant activity, i.e., higher parasite load leads to intense immune response and decrease of antioxidant activity [146,148]. In this regard, there is a tendency to decrease the levels or even deplete GSH turning liver more vulnerable for adverse effects of ROS and other parasitic metabolites in the course of infection [148]. It has been described that patients treated with anthelmintic leading to eradication of worms are still unable to reverse hepatic fibrosis. Since morbidity associated to schistosomiasis are mainly resulted of liver fibrosis most interest has been focus on compounds that are capable to stimulate not only synthesis of antioxidant enzymes as well as enzymes associated to liver function. Administration of antioxidants on experimentally *S. mansoni* infected mice revealed that they are capable to restore activity of antioxidant and liver enzymes nearly to levels detected on controls [111,115,116,120,121,125,128,135]. Generally, increasing of antioxidant enzymes activity is

accompanied by reduction on granulomas size and number which consequently improve liver architecture and functions [111,119,123,127,129,137,140]. The protective effects of antioxidants in some subcellular compartments may be due to its indirect antioxidant actions, e.g. stimulation of enzymes that promote the synthesis of other antioxidants or metabolize reactive species to non-radical products. On the other hand, parasites have developed antioxidant enzyme system, similar to humans, to defend themselves against ROS generated in immune host attack. In *S. mansoni* SOD, GR, GPX, CAT, glutathione-s-transferase (GST), thioredoxin reductase (TrxR) are major antioxidant enzymes that are involved in detoxification processes [149]. Therefore, the antioxidant defense mechanism of adult worms may represent potentially good target chemotherapy. It has been demonstrated that antioxidants, such as those present in extracts of *N. sativa* and curcumin (Figure 7), are able to inhibit parasitic antioxidant enzymes as well enzymes related to glucose metabolism (hexokinase, HK and glucose-6-phosphate, G-6-PDH) culminating in increase of oxidative stress that could turn render the parasite vulnerable to damage by host immune attack [131,137].

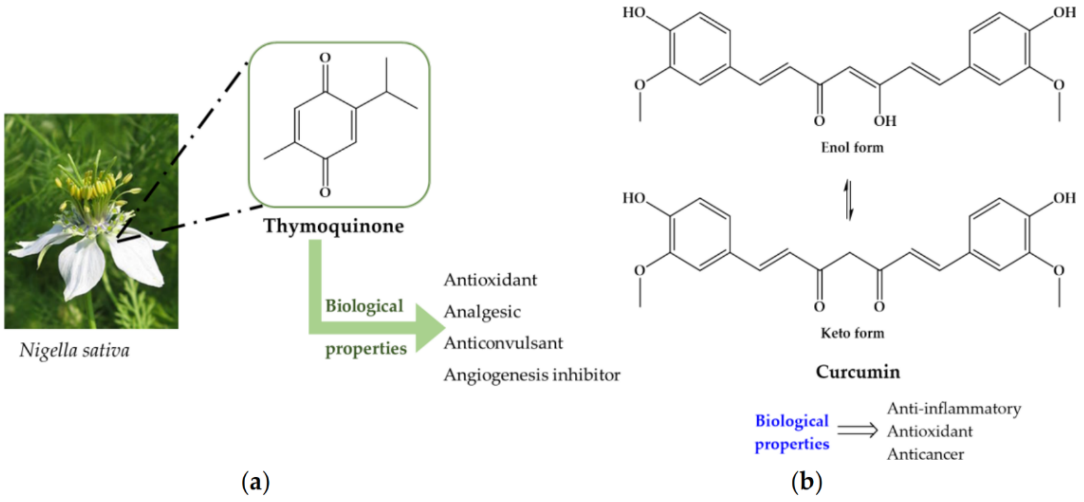


Figure 7. (**a**) *Nigella sativa* and its therapeutically active constituent, thymoquinone, responsible for diverse biological properties; (**b**) Chemical structures of curcumin and its biological properties. Curcumin is the principal curcuminoid of *Curcuma longa*. It is a diarylheptanoid, which is a natural phenol that exists in enolic form in organic solvents and as a keto form in water.

Besides antischistosomal activity and ability to restore liver functions, antioxidants modulate and immunomodulatory response promoting alteration in some cytokines [122,126,133,136,139]. Cytokines play an important role in immunomodulation during schistosomiasis. There are several events that are determined by a balance between different immune responses modulate by certain cytokines which are directed both against larval and adult stages of the parasite, as well as parasite eggs trapped in the tissues [150]. Eggs trapped in tissue secrete release antigens which are taken up by macrophages that stimulate T helper cells to secrete tumor necrosis factor α (TNF-α), which in turn drive to cell-mediate response attracting more immune cells around ova. As the granuloma becomes more organized, the T helper cells, produce different interleukins (IL) completing granuloma maturation towards the late stage of granuloma formation [150]. In case of murine schistosomiasis, TNF-α, IL-1, IL-2 and IL-12 are the causative IL in granuloma formation. In addition, granuloma cells comprise of macrophages, lymphocytes, eosinophils and release of profibrotic lymphokines such as IL4 [151] that stimulates fibroblasts to secrete collagen and other matrix proteins [152]. In experimentally *S. mansoni* infected mice treated with antioxidants shown controversial results, while treatment with curcumin resulted in low serum level of both IL-12 and TNF-α, *N. sativa* combined with ART or PZQ and *Allium sativum* showed significant increase on IL-2, IL-12 and TNF-α [127,133,136]. The dissimilarity of results obtained might be related to sampling time of experiment performed by Sheir and colleagues, which occurs after granuloma formation [131]. Moreover,

treatment with *N*-acetylcysteine (NAC, Figure 8) increase synthesis of IL-10 which regulates the synthesis of several pro-inflammatory cytokines and is considered an efficient inhibitor of INF-γ, IL-12 and IL-4 [139].

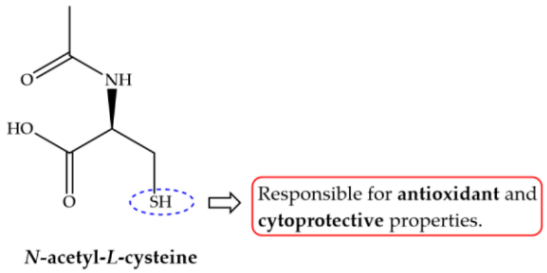


Figure 8. *N*-acetyl-L-cysteine (NAC) displays antioxidant and cytoproctive properties that are related to its free sulfhydryl group (blue elipse) which directly react with electrophiles including reactive radicals.

Therefore, these responses produced by antioxidants might explain their role in reduce size and number of granuloma in infected host. Also, immunoglobins IgG and IgM have been shown to have a pivotal role in humoral response to schistosomal infection being related to periportal fibrosis and portal hypertension in patients with advanced schistosomiasis mansoni [153,154]. Administration of antioxidants to *S. mansoni* infected mice reduce serum IgG and IgM which might be directly linked to reduction of granulomas [127].

Since parasitic antigens induce a host immune response, diverse *S. mansoni* antigens including adult worm antigen (SWAP), cercarial antigen (CAP) among other have been used to immunized experimental animals against *S. mansoni* either alone, in combination or with adjuvants. Unfortunately, most of studies have not achieved even a low significant protection against schistosome infection [155–157]. Immunization using SWAP and CAP alone or concurrently with melatonin (Figure 9) demonstrated that antioxidant enhanced SWAP efficacy which was confirmed by the absence of significant antibody (Ab) response in group immunized with SWAP + melatonin [120]. In different studies, it was also demonstrated that treatment with antioxidants augmented IgG response against SWAP, CAP and soluble egg antigen (SEA) [114,135]. These findings indicate the early and continuous antioxidant administration is responsible for the immunoprophylactic effect and may protect the liver against infection by reducing worm burden leading to improvement of liver function.

Generally, administration of antioxidants mixed or combined with antischistosomal drugs such as PZQ and ART, improved the parasitological and biochemical parameters described [115,123,125,132,133,138–140]. Considering that compounds present different mode of action it is reasonable hypothesized that those improvements are related with synergistic and/or cumulative effects of compounds when administrated in combination.

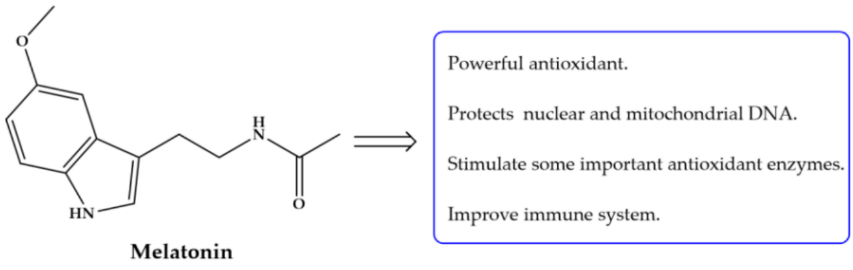


Figure 9. Melatonin (MEL) is a powerful antioxidant with a noteworthy protective action for nuclear and mitochondrial DNA due to efficacy as a radical scavenger. MEL may also stimulate activity of some key enzymes that participate in immunological functions.

Remarkably, antioxidants present not only antischistosomal activity but also induce restoration of organ target functions and improved host immunity, at least in animal model. Therefore, they should be considered as adjuvants in combine treatment of schistosomiasis. Nevertheless, many studies are required to fully understand the exact mechanism of antioxidants alone or combined against schistosomiasis. Additionally, clinical trials are required in order to assess if these results in animal model are reproduced in human host.

Interestingly, studies related to the effect of antioxidant against schistosomiasis haematobia are scarce. Despite there is no reliable animal model for this infection, it is extremely important conduct novel investigations of new therapeutic approaches against disease. *S. haematobium* is considered a biological carcinogen and in fact, the most adverse pathology associated to infection is bladder cancer [12]. Recently, it has been hypothesized about the role of parasitic reactive electrophilic compounds, e.g., estrogen-like metabolites, on initiation of squamous cell carcinoma (SCC) [10,158–160]. Possibly, these metabolites are capable to react with host DNA leading to formation of DNA-adducts and liberation of ROS, triggering a cascade of events that ultimately leads to development of SCC. Some evidences point out that antioxidants can prevent DNA damage [161] and block-cancer initiating process in case of breast cancer [162]. Therefore, it should be informative to assess their effect, alone or combine, in counteracting formation of these parasitic reactive metabolites.

5. Concluding Remarks

Nowadays, mass drug administration is main strategy for control of schistosomiasis but relies on the effectiveness of a single drug, PZQ. Although PZQ is highly effective, given by mouth and relatively inexpensive, PZQ has shortcomings that include lack of activity against immature schistosomes [1,17,19]. Moreover, PZQ alone does not lead to resolution of the histopathological damage characteristic of chronic schistosomiasis. Hence there is a need for new strategies, targeting not only parasite but also infection-associated pathogenesis. For the last years several combinations among different agents with PZQ and/or antimalarial and others are reported to represent encouraging leads for treatment approaches to overcome limitations of PZQ monotherapy (Figure 10a). Whereas PZQ combined with antioxidant agents (Figure 10) might or might not alter PZQ efficacy, combinations may nonetheless ameliorate tissue damage and infection-associated complications. Even though some antioxidants failed to inflict obvious harm to the schistosomes, they markedly reduced granulomatous inflammatory reactions as well as improved antioxidant and immunological responses to the infection. Alone or combined with other drugs, antioxidants might be valuable adjuvants to reduce morbidity and mortality of schistosomiasis. Moreover, natural antioxidants are considered safe for human use. Attempting new combinations of anthelmintic drugs with other biomolecules such as antioxidants provides new avenues for discovery of alternatives to PZQ.

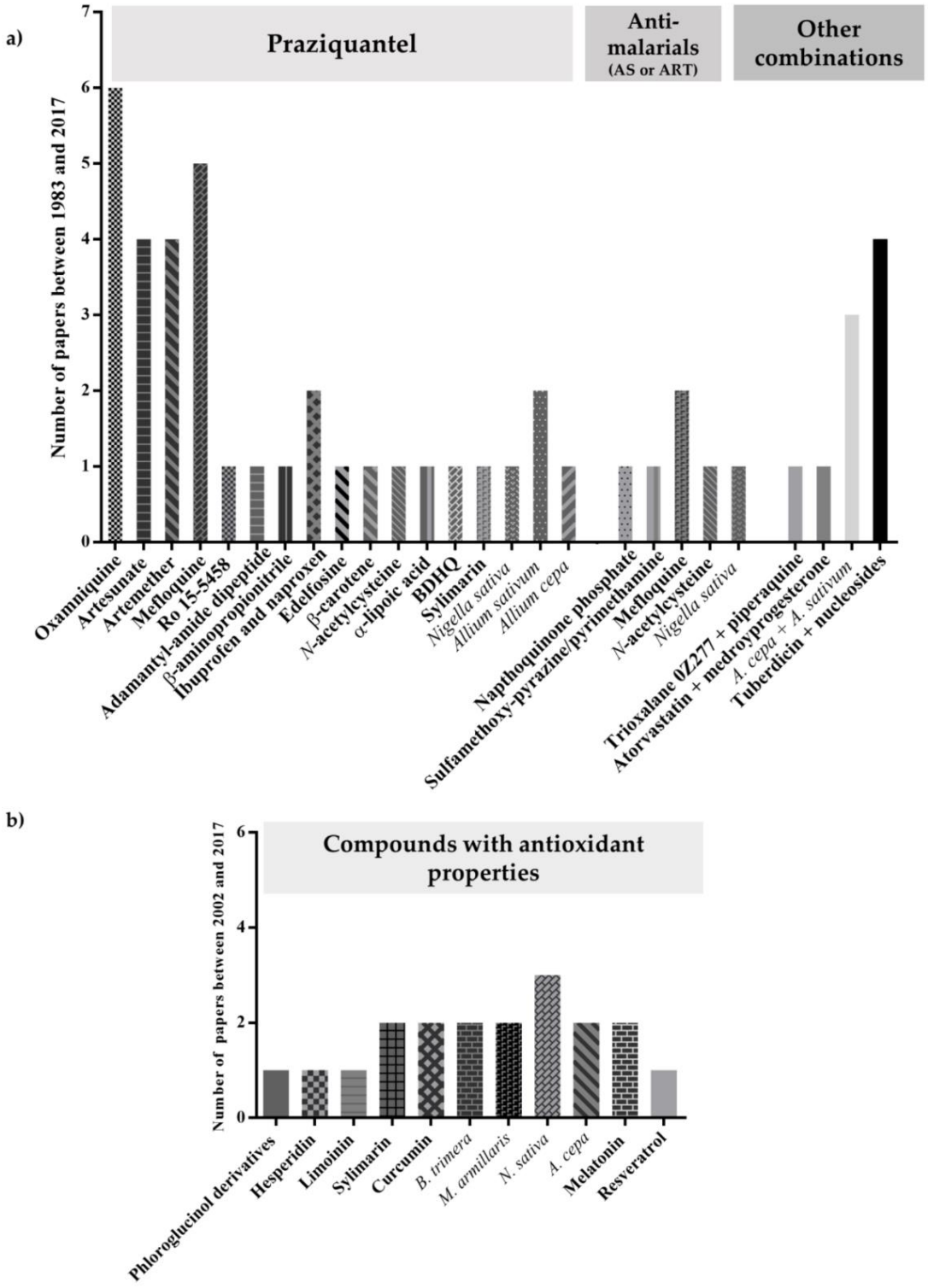


Figure 10. Graphical depiction of the number of publications dealing with (**a**) combinations between compounds with PZQ, antimalarials and other combinations; (**b**) compounds with antioxidants alone performed on both in vitro on in vivo cultured forms of schistosomes.

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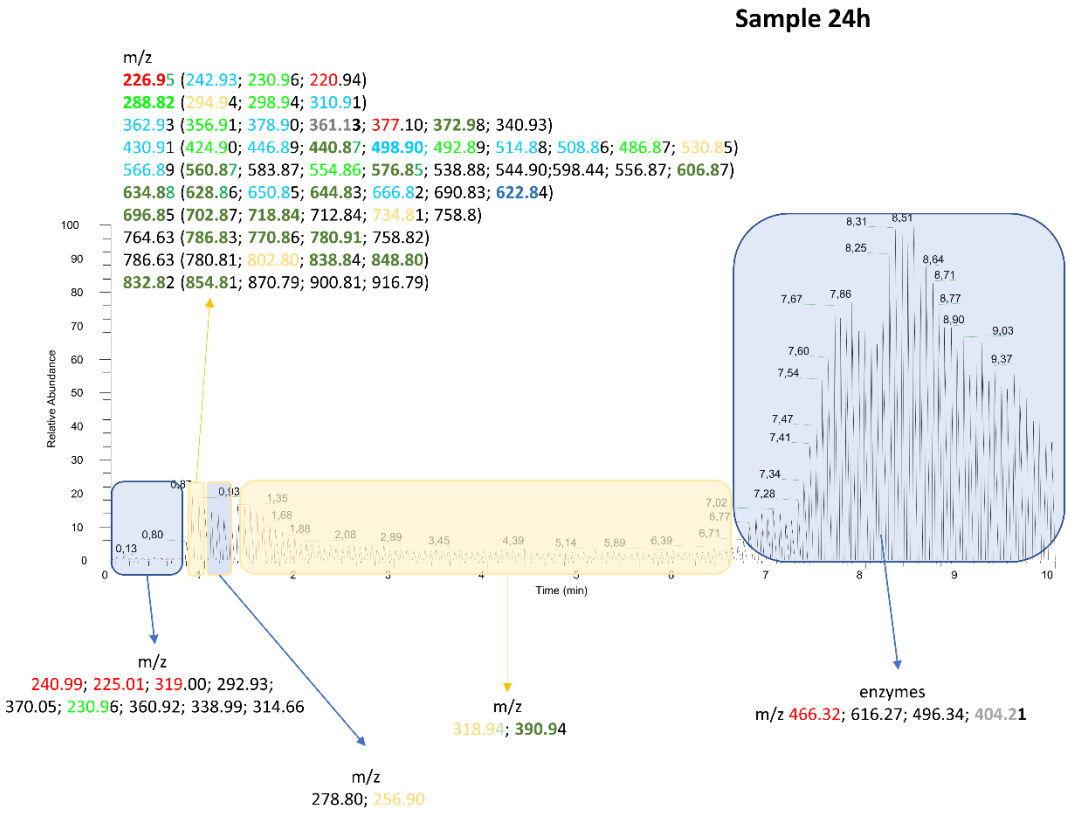
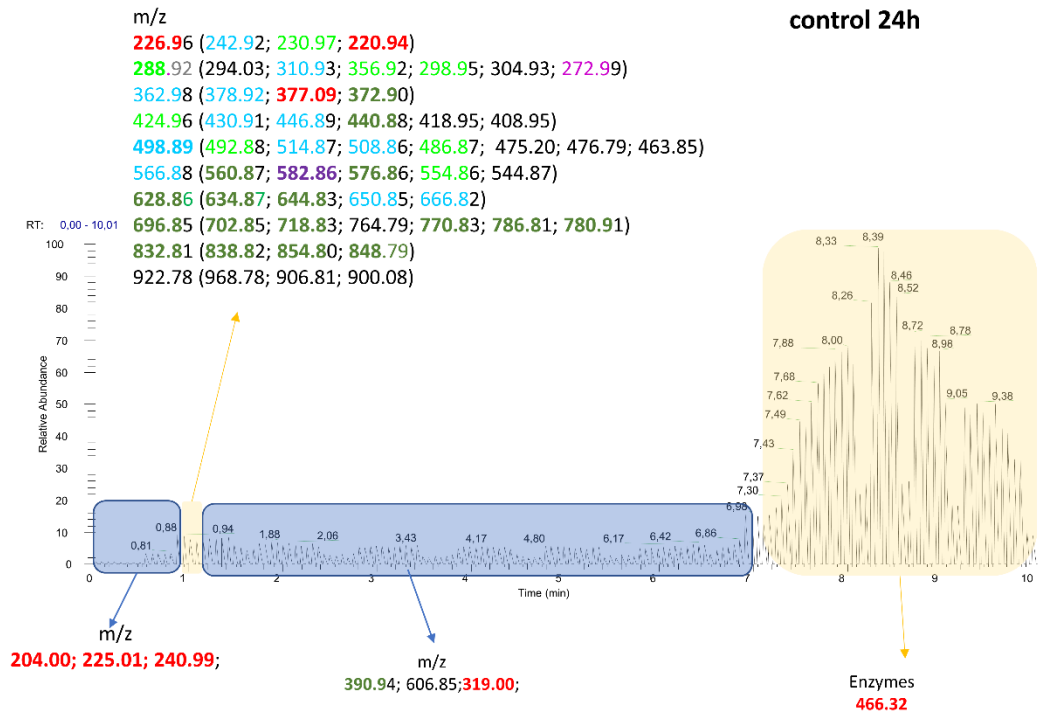
Appendix 4

Support information of scientific article presented in Section III - Chapter 3

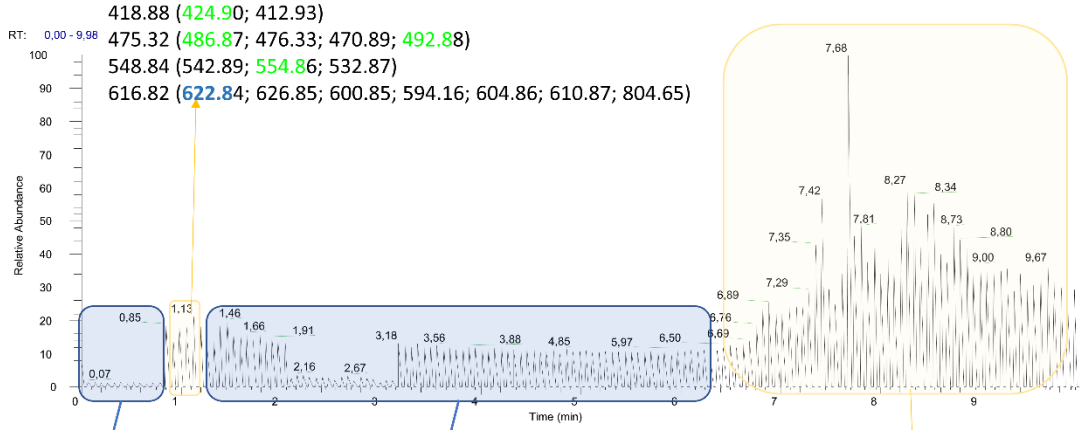
Additional file 1. Chromatograms obtained by LC-MS/MS of different aliquots analyzed. (.pptx)





Sample 72h

m/z
230.96 (**226.96**; **220.93**; 249.16; 214.99)
298.95 (288.92; 272.94; 265.11)
361.13 (**377.10**; 356.91; 402.90)
418.88 (424.90; 412.93)
475.32 (486.87; 476.33; 470.89; 492.88)
548.84 (542.89; 554.86; 532.87)
616.82 (**622.84**; 626.85; 600.85; 594.16; 604.86; 610.87; 804.65)



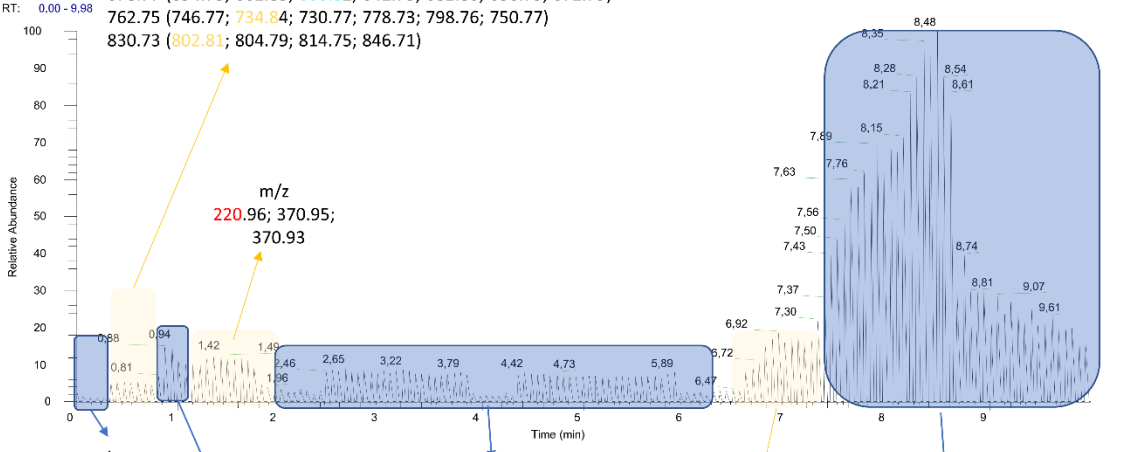
m/z
204.00; 225.47; **319.00**

319 (361.13; **377.10**; 356.10; 418.88)

enzymes
m/z **466.32** e 404.21

Control 72h

m/z
226.95 (242.93;248.92; 258.90; 232.92;240.98; 264.93);
316.68 (310.92; 300.90; 294.92; 326.91; 278.91; 332.85);
378.90 (384.86; 368.87; 394.87; 362.92; 400.84; 361.10; **377.09**);
446.88 (452.85; 436.86; 462.86; 468.83; 430.90; 458.82; 420.85; 478.84)
520.86 (514.87; 530.85; 536.82; 542.79; 504.85; 562.80; **498.89**; 558.78; 552.79; 546.82; 510.82; 566.88)
610.78 (594.80; 598.83; **582.86**; 626.77; 588.82; 650.85)
678.77 (694.75; 662.85; 666.82; 642.75; 682.80; 656.79, 672.79)
762.75 (746.77; 734.84; 730.77; 778.73; 798.76; 750.77)
830.73 (802.81; 804.79; 814.75; 846.71)



m/z
220.96; 370.95;
370.93

m/z
240.99; **204.00**; **225.01**

m/z
319.00

m/z
256.90; 318.94; 370.93;
354.89; 474.85; 452.86; 508.87

m/z
Between 900 and 1000

enzymes
m/z **466.32**; 304.30; 404.21

Additional file 2. All m/z detected during analysis of LC-MS/MS of aliquots of samples and control during the course of reaction. Common and exclusive m/z detected for each of aliquots. (.pptx)

All m/z detected

	(+ CYP)	(- CYP)
	S24h	**C24h**
m/z	240.99; 225.01; 319.00; 292.93; 370.05; 230.96; 360.92; 338.99; 314.66; 226.95 (242.93; 230.96; 220.94); 288.82 (294.94; 298.94; 310.91); 362.93 (356.91; 378.90; 361.13; 377.10; 372.98; 340.93); 430.91 (424.90; 446.89; 440.87; 498.90; 492.89; 514.88; 508.86; 486.87; 530.85); 566.89 (560.87; 583.87; 554.86; 576.85; 538.88; 544.90;598.44; 556.87; 606.87); 634.88 (628.86; 650.85; 644.83; 666.82; 690.83; 622.84); 696.85 (702.87; 718.84; 712.84; 734.81; 758.8); 764.63 (786.83; 770.86; 780.91; 758.82); 786.63 (780.81; 802.80; 838.84; 848.80); 832.82 (854.81; 870.79; 900.81; 916.79); 278.80; 256.90; 318.94; 390.94; 466.32 (77)	204.00; 225.01; 240.99; 226.96 (242.92; 230.97; 220.94); 288.92 (294.03; 310.93; 356.92; 298.95; 304.93; 272.99); 362.98 (378.92; 377.09; 372.90); 424.96 (430.91; 446.89; 440.88; 418.95; 408.95); 498.89 (492.88; 514.87; 508.86; 486.87; 475.20; 476.79; 463.85); 566.88 (560.87; 582.86; 576.86; 554.86; 544.87); 628.86 (634.87; 644.83; 650.85; 666.82); 696.85 (702.85; 718.83; 764.79; 770.83; 786.81; 780.91); 832.81 (838.82; 854.80; 848.79); 922.78 (968.78; 906.81; 900.08); 390.94; 606.85; 319.00; 466.32
	S72h	**C72h**
m/z	204.00; 225.47; 319.00; 230.96 (226.96; 220.93; 249.16; 214.99); 298.95 (288.92; 272.94; 265.11); 361.13 (377.10; 356.91; 402.90); 418.88 (424.90; 412.93); 475.32 (486.87; 476.33; 470.89; 492.88); 548.84 (542.89; 554.86; 532.87); 616.82 (622.84; 626.85; 600.85; 594.16; 604.86; 610.87; 804.65); 319.00 (361.13; 377.10; 356.10; 418.88); 466.32; 404.21	240.99; 204.00; 225.01; 226.95 (242.93; 248.92; 258.90; 232.92; 240.98; 264.93); 316.68 (310.92; 300.90; 294.92; 326.91; 278.91; 332.85); 378.90 (384.86; 368.87; 394.87; 362.92; 400.84; 361.10; 377.09); 446.88 (452.85; 436.86; 462.86; 468.83; 430.90; 458.82; 420.85; 478.84); 520.86 (514.87; 530.85; 536.82; 542.79; 504.85; 562.80; 498.89; 558.78; 552.79; 546.82; 510.82; 566.88); 610.78 (594.80; 598.83; 582.86; 626.77; 588.82; 650.85); 678.77 (694.75; 662.85; 666.82; 642.75; 682.80; 656.79, 672.79); 762.75; (746.77; 734.84; 730.77; 778.73; 798.76; 750.77); 830.73 (802.81; 804.79; 814.75; 846.71); 319.00; 466.32; 304.30; 404.21 (68)

Common:

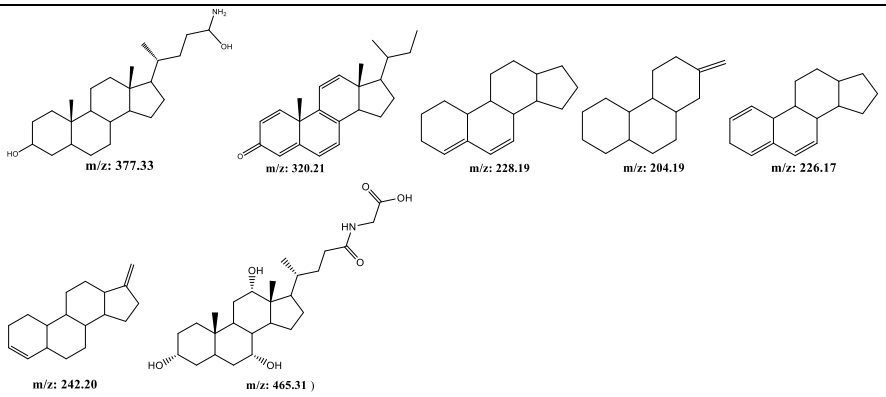
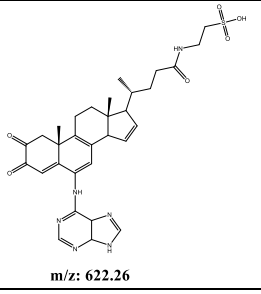
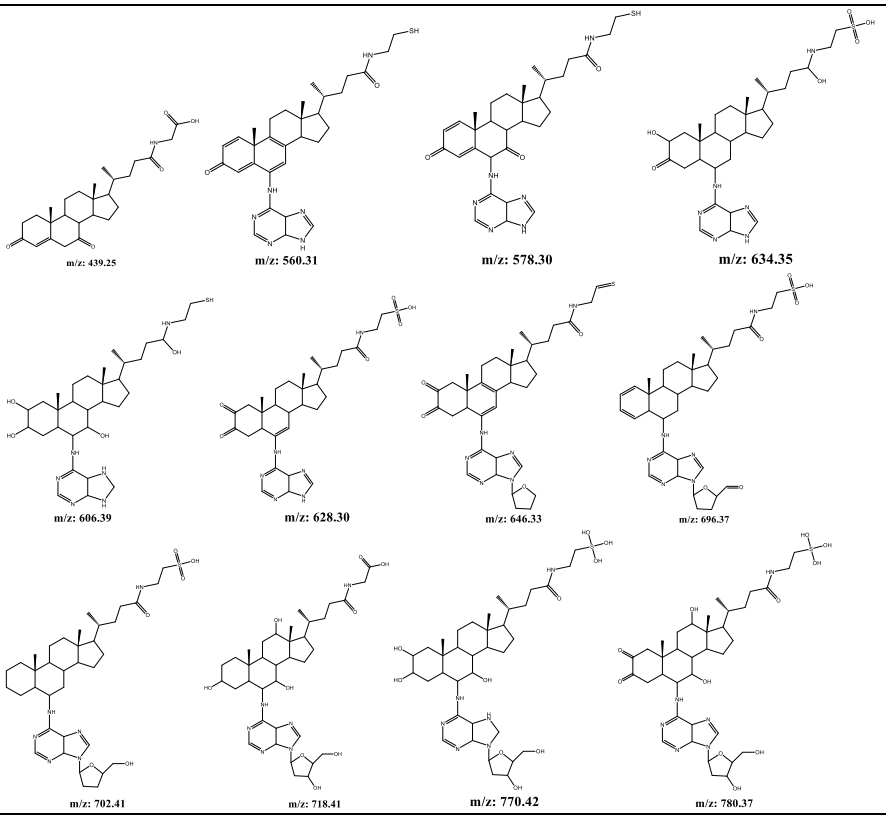
- to all aliquots
- S24, C24h, C72h;
- S24h, S72h, C24h;
- S24h, C24h;
- S24, C72h
- S72h, C72h
- S24h, S72h
- S72h, C24h
- C24h, C72h
- S72h, C72h

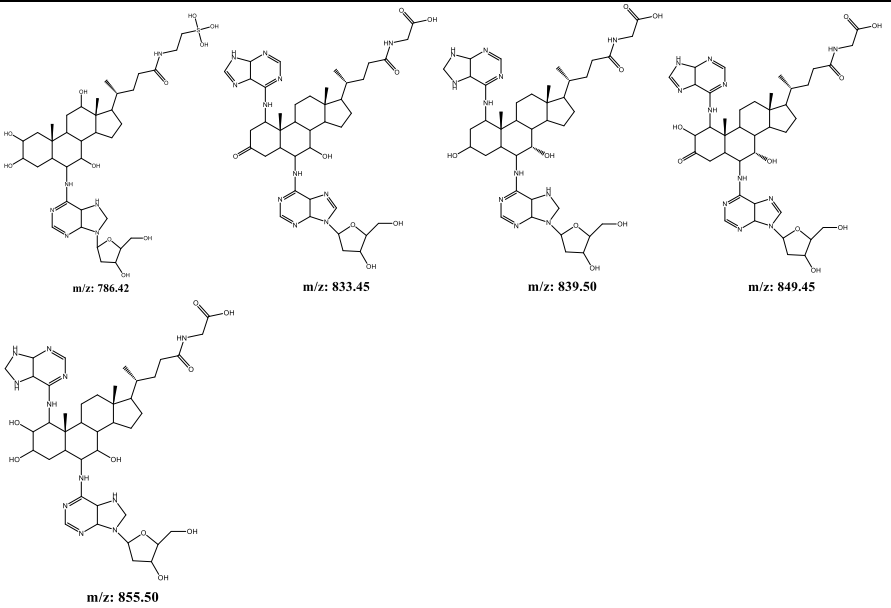
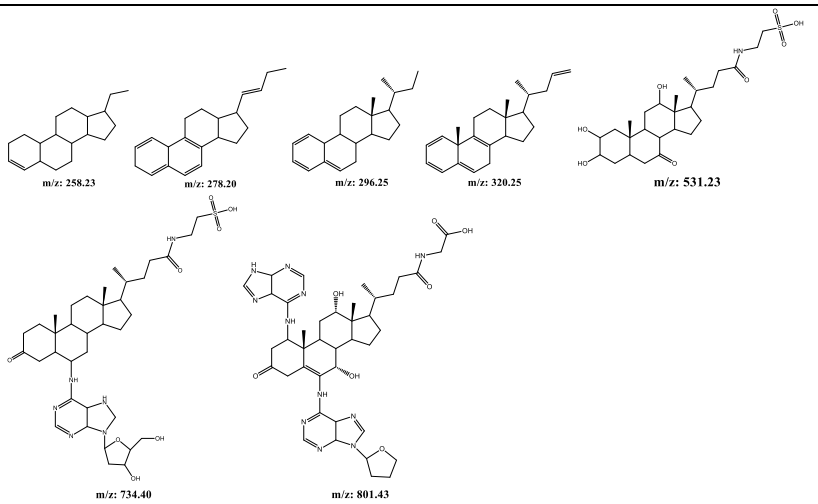
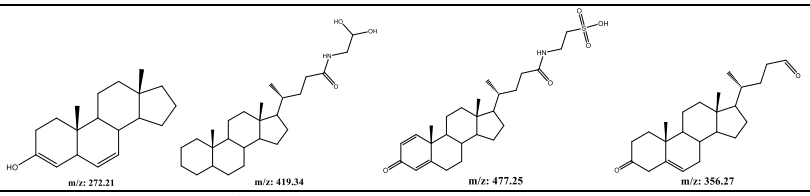
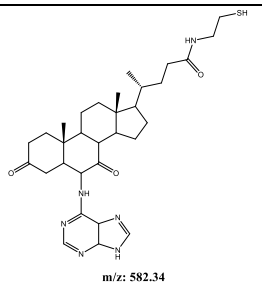
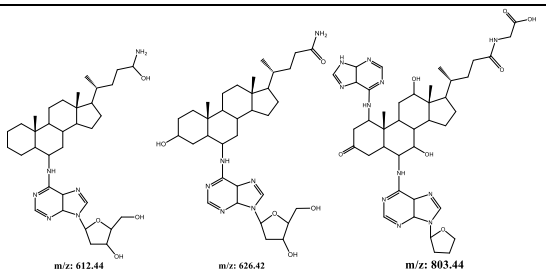
S- sample
C- control

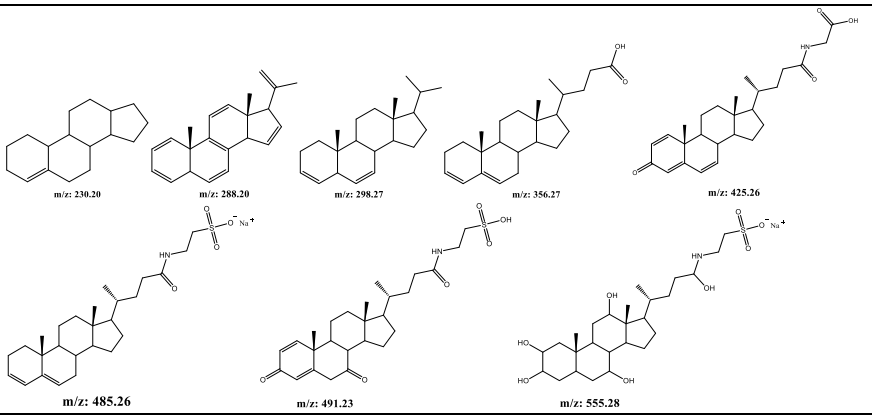
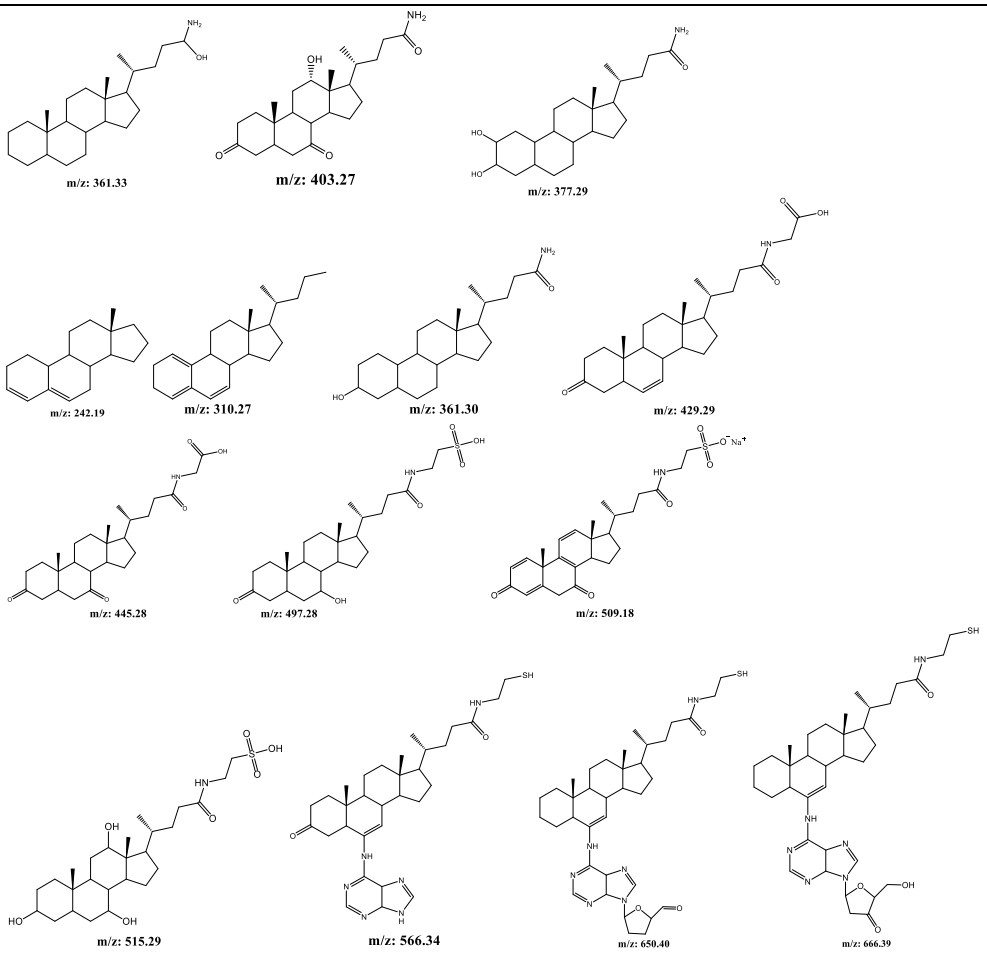
Exclusive

	(+ CYP)	(- CYP)
	S24h	**C24h**
m/z	292.93; 370.05; 360.92; 338.99; 314.66; 340.93; 583.87; 544.90; 598.44; 556.87; 690.83; 758.8; 764.63; 786.63; 870.79; 900.81; 916.79; 278.80; 616.27; 496.34	294.03; 304.93; 408.95; 476.79; 463.85; 544.87; 922.78 968.78; 906.81; 900.08
	S72h	**C72h**
m/z	249.16; 214.99; 265.11; 412.93; 476.33; 470.89; 548.84; 532.87; 616.82; 626.85; 600.85; 594.16; 604.86; 610.87; 804.65; 225.47; 356.10;	248.92; 258.90; 232.92;240.98; 264.93; 300.90; 326.91; 278.91; 332.85; 384.86; 368.87; 394.87; 400.84; 452.85; 436.86; 462.86; 468.83; 458.82; 420.85; 478.84; 520.86; 536.82; 542.79; 504.85; 562.80; 558.78; 552.79; 546.82; 510.82; 610.78, 594.80; 598.83; 626.77; 588.82; 678.77; 694.75; 662.85; 642.75; 682.80; 656.79, 672.79; 762.75; 746.77; 730.77; 778.73; 798.76; 750.77; 830.73; 804.79; 814.75; 846.71; 370.93; 354.89; 474.85; 452.86; 304.30;

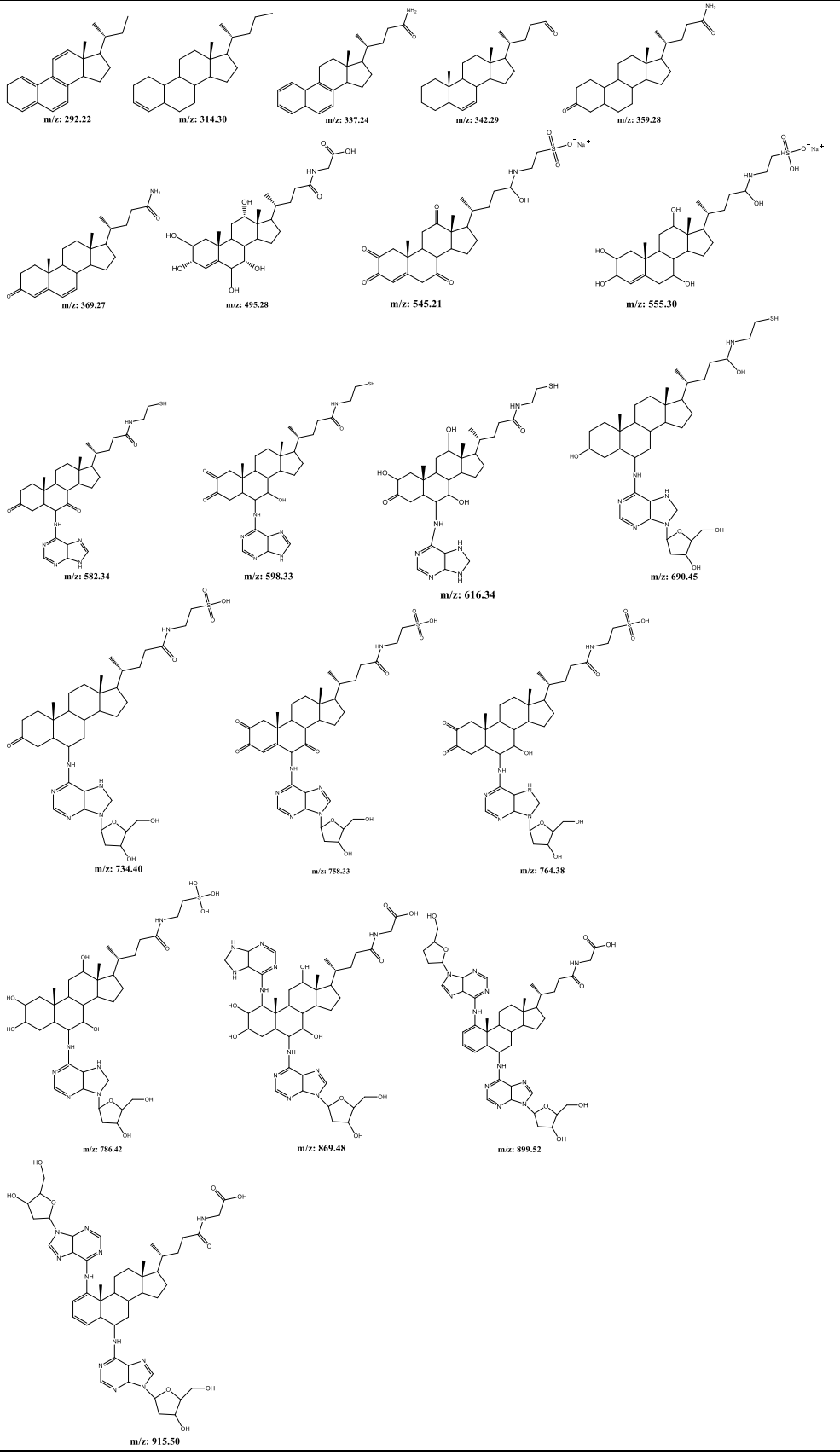
Additional file 3. Postulated structures for common compounds to different aliquots. (.docx)

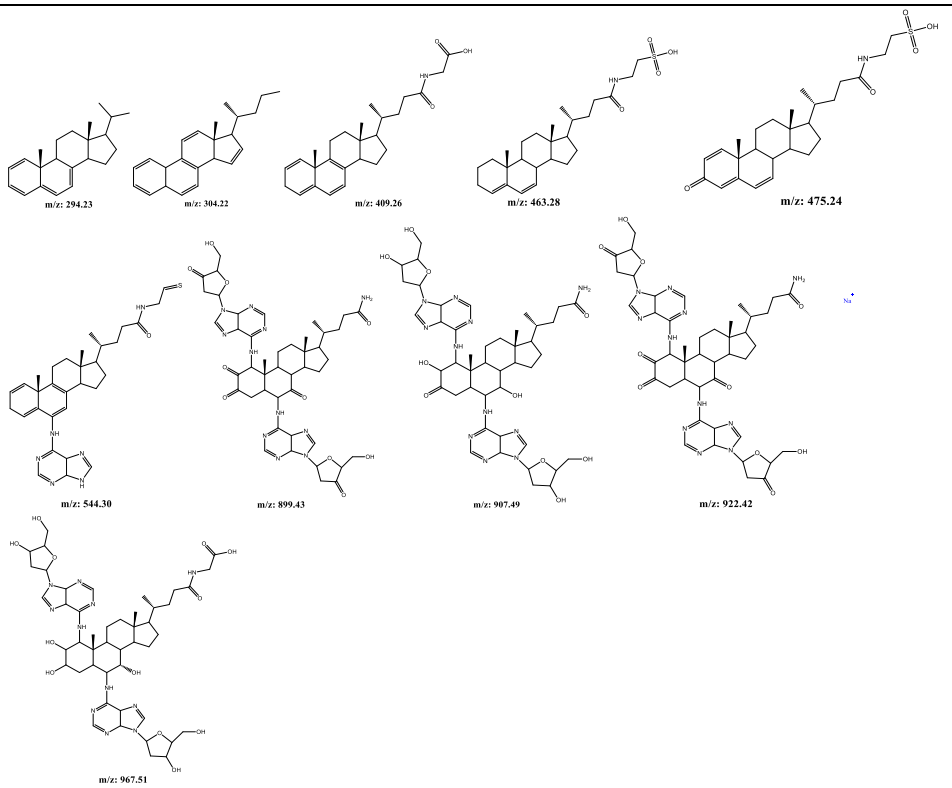
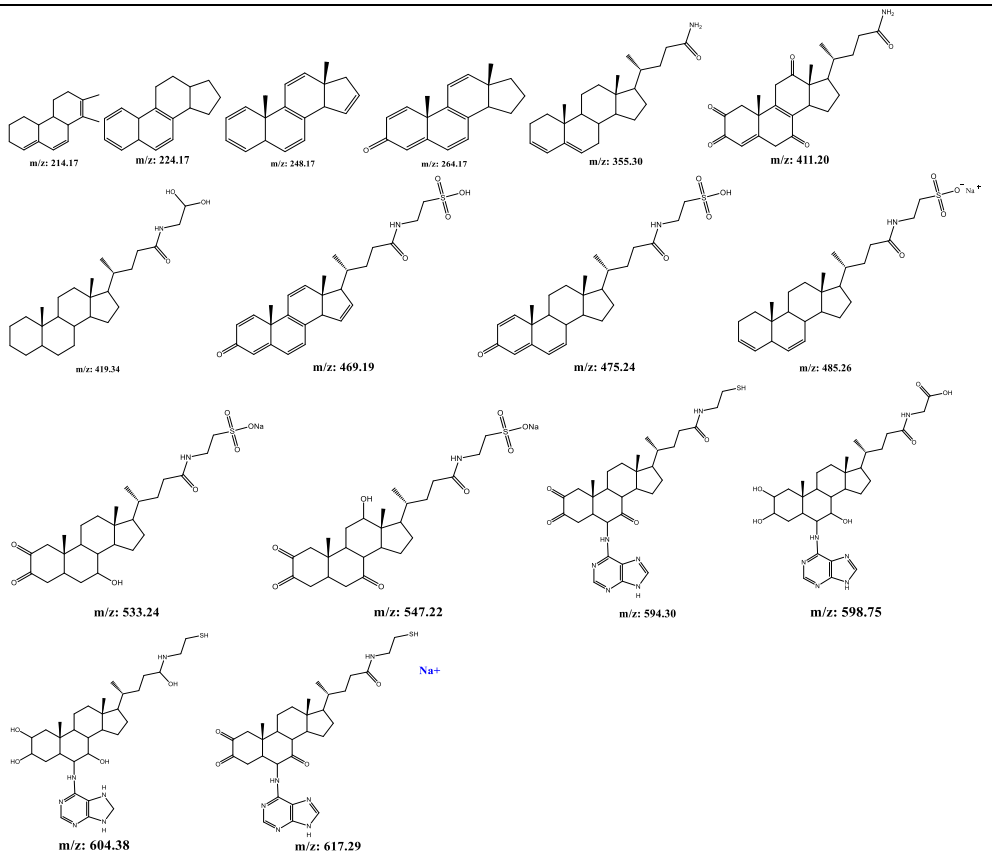
Common	Postulated structures
All aliquots	
S24h and A72h	
S24h and C24h	

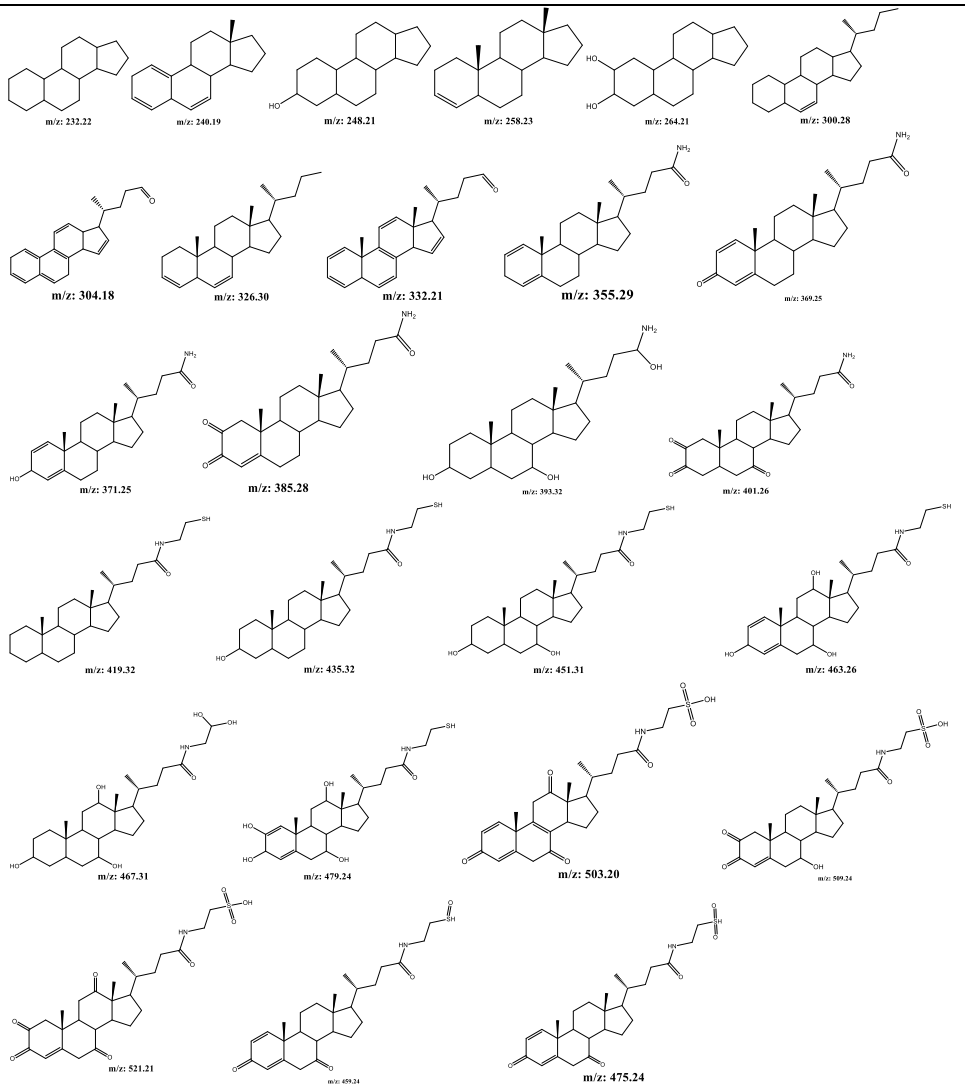
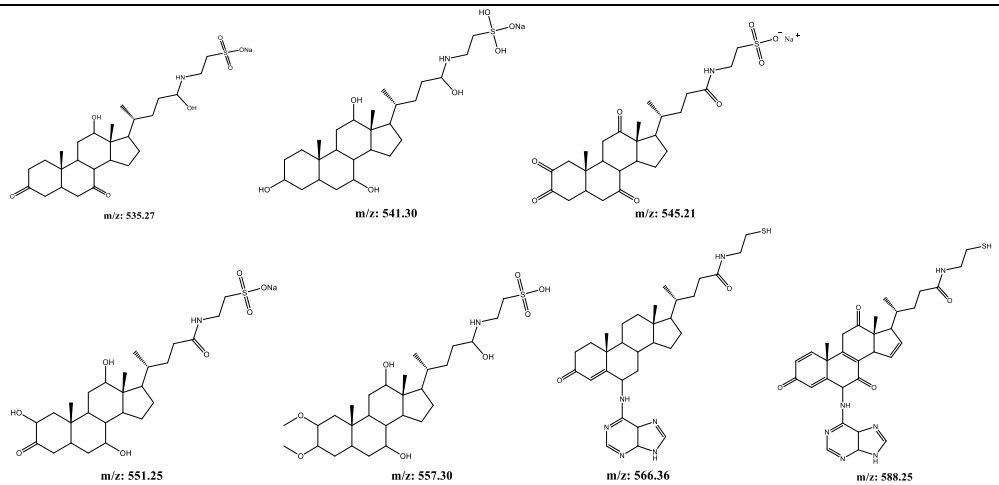
	
C72h and S24h	
C24h and S72h	
C72h and C24h	
S72h and C72h	

S24h, S72h, C24h	
S72h, S24h, C72h	
C24h, C72h, S72h	Not found.

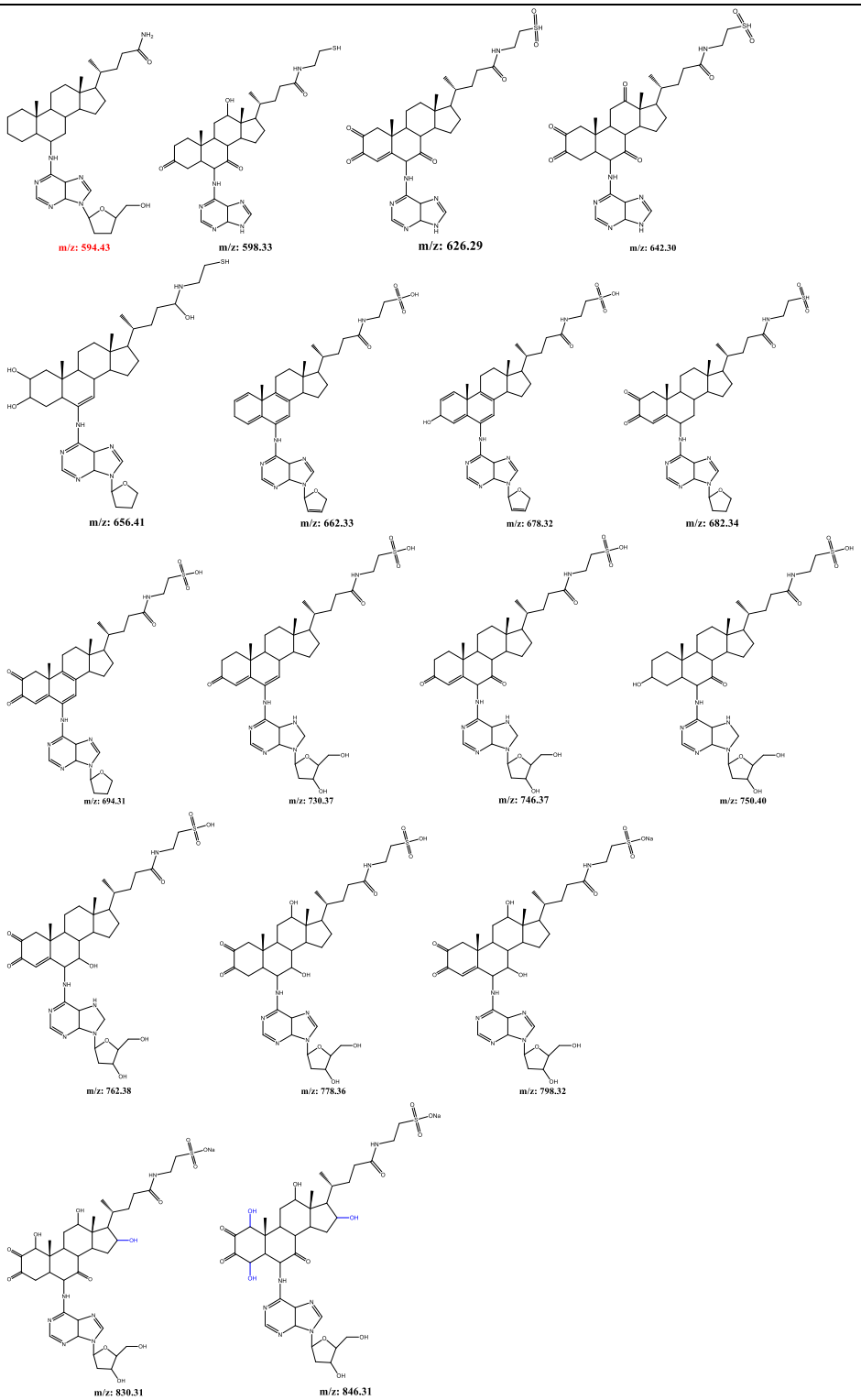
Additional file 4. Postulated structures for exclusive m/z detected on samples and control during course of reaction. (.docx)

Exclusive	Postulated structures
S24h (+ CYP)	

C24h (-CYP)	
S72h (+ CYP)	

Exclusive	Postulated structures
C72h (- CYP)	
C72h (- CYP	

C72h (- CYP) (cont.)



Appendix 5

Support information of scientific article presented in Section III - Chapter 6

Table S1. Oviposition expressed as the number of eggs deposited per worm during in *vitro* assay.

	1h	17h	24h	48h	72h
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Compounds alone					
control	6.0 ± 0.0	29.3 ± 0.6	29.7 ± 0.6	70.7 ± 0.6	108.3 ± 2.3
DMSO 0.1%	107.0 ± 13.3	137.2 ± 39.2	161.5 ± 36.2	263.7 ± 4.7	383.5 ± 79.9
PZQ	26.7 ± 1.5	27.7 ± 1.5	29.7 ± 1.5	27.3 ± 2.1	29.3 ± 1.5
AS	50.3 ± 1.5	71.3 ± 1.5	73.3 ± 2.5	73.0 ± 2.0	75.7 ± 2.5
FBZ	33.7 ± 1.2	39.0 ± 1.7	31.7 ± 1.2	41.7 ± 1.5	41.7 ± 1.5
TMT	41.3 ± 0.6	41.0 ±1.0	43.0 ± 1.0	42.7 ± 1.2	42.7 ± 1.2
VDT	4.3 ± 1.5	4.7 ± 1.5	4.0 ± 1.0	4.3 ± 0.6	5.0 ± 2.0
IMT	30.7 ± 2.1	34.0 ± 2.0	34.7 ± 1.5	36.3 ± 1.5	36.0 ± 3.0
Resv	17.3 ± 0.6	17.3 ± 1.5	34.3 ± 3.1	34.3 ± 3.5	34.3 ± 3.1
NAC	59.3 ± 3.8	138.7 ± 2.5	173.0 ± 3.6	261.0 ± 2.0	322.0 ± 2.6
Flav	85.7 ± 0.6	88.0 ± 8.2	88.0 ± 8.2	88.0 ± 2.6	91.3 ± 1.5
Mel	69.7 ± 0.6	145.7 ± 3.5	192.3 ± 4.9	249.7 ± 1.5	307.0 ± 2.6
DiPept	41.3 ± 2.1	98.0 ± 1.0	105.7 ± 1.5	127.3 ± 2.5	167.0 ± 2.0
OXA	2.7 ± 0.6	3.0 ± 1.0	3.3 ± 0.6	3.3 ± 1.5	3.3 ± 1.5
Curc	52.7 ± 1.5	52.7 ± 1.5	52.7 ± 1.5	52.7 ± 1.5	52.7 ± 1.5
Drug+AntiOx					
PZQ+Resv	58.7 ± 1.5	60.3 ± 2.1	61.3 ± 1.5	61.0 ± 2.0	61.3 ± 1.5
AS+Resv	55.0 ± 2.0	56.3 ± 1.5	61.3 ± 2.3	61.7 ± 1.5	61.7 ± 2.3
AS+NAC	59.3 ± 2.1	75.3 ± 3.8	76.0 ± 2.6	76.3 ± 1.5	76.7 ± 1.5
AS+DiPept	50.7 ± 3.4	70.7 ± 1.2	72.3 ± 3.8	88.0 ± 1.0	88.0 ± 1.0
AS+OXA	4.7 ± 0.6	4.7 ± 0.6	4.7 ± 0.6	4.7 ± 0.6	4.7 ± 0.6
AS+Curc	49.3 ± 2.1	49.0 ± 2.6	49.0 ± 2.6	49.0 ± 2.6	49.0 ± 2.6
AS+Flav	43.3 ± 1.2	53.3 ± 1.2	53.3 ± 1.2	53.3 ± 1.2	53.3 ± 1.2
AS+Mel	16.7 ± 1.2	23.0 ± 1.0	23.0 ± 1.0	23.0 ± 1.0	23.0 ± 1.0
FBZ+Flav	41.7 ± 1.1	42.3 ± 2.1	47.7 ±1.2	47.0 ± 1.5	58.7 ± 4.0
FBZ+Mel	83.0 ± 2.0	127.0 ± 0.6	154.7 ± 4.9	155.3 ± 7.4	158.3 ± 1.2
IMT+Flav	57.7 ± 1.1	58.7 ± 1.2	57.0 ± 1.0	57.7 ± 1.5	57.3 ± 1.5
IMT+Mel	67.0 ± 1.0	71.7 ± 1.5	71.7 ± 1.5	71.7 ± 1.5	71.7 ± 1.5
TMT+Mel	22.3 ± 1.5	26.3 ± 2.1	25.0 ± 1.0	25.0 ± 1.0	25.0 ± 1.0
TMT+Flav	69.3 ± 0.6	71.0 ± 1.0	71.7 ± 1.5	71.7 ± 1.5	69.3 ± 1.2
VDT+Resv	28.0 ± 1.7	31.0 ± 1.7	31.0 ± 1.7	31.0 ± 1.7	33.7 ± 3.2
VDT+Flav	43.3 ± 2.1	43.3 ± 2.1	43.3 ± 2.1	43.3 ± 2.1	43.3 ± 2.1
AntiOx+AntiOx					
OXA+Curc	24.0 ± 1.7	24.0 ± 1.7	24.0 ± 1.7	24.0 ± 1.7	24.0 ± 1.7
Flav+NAC	61.7 ± 3.1	59.0 ± 1.0	65.0 ± 2.0	62.3 ± 0,6	80.3 ± 1.5
Flav+Mel	29.3 ± 1.2	32.7 ± 1.5	32.0 ± 2.0	40.3 ± 0,6	40.3 ± 2.5
Flav+DiPept	57.7 ± 0.6	72.7 ± 1.2	73.7 ± 0.6	73.0 ± 2.0	81.0 ± 2.6
Mel+Dipept	44.7 ± 1.5	86.0 ± 3.6	111.0 ± 3.0	152.0 ± 1.7	217.0 ± 9.8
Mel+NAC	52.3 ± 1.2	111.3 ± 0.6	143.0 ± 2.6	171.7 ± 1.2	205.0 ± 2.0
Dipept+NAC	38.0 ± 2.0	73.7 ± 0.6	86.7 ± 0.6	95.0 ± 1.0	137.3 ± 1.5

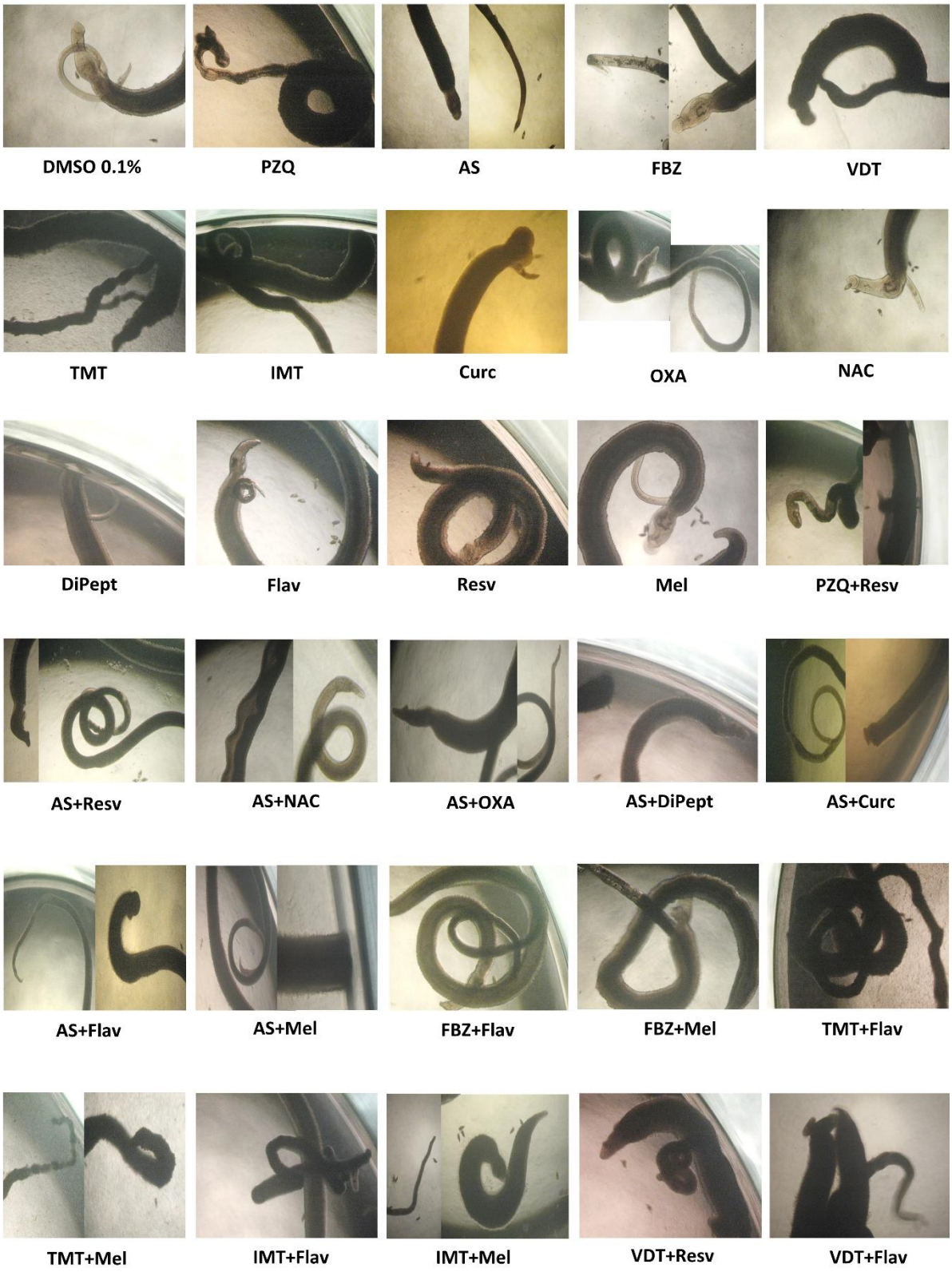
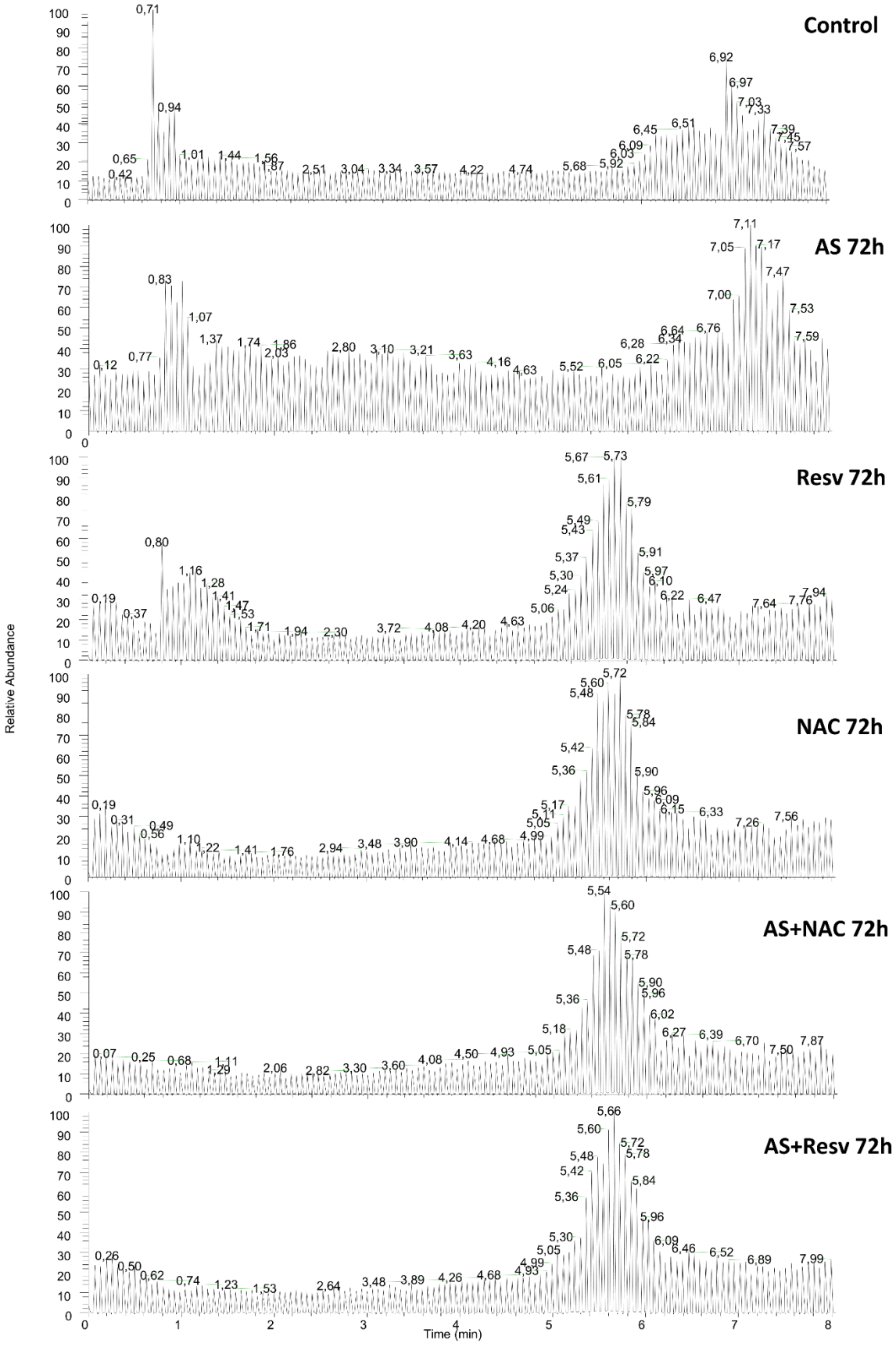


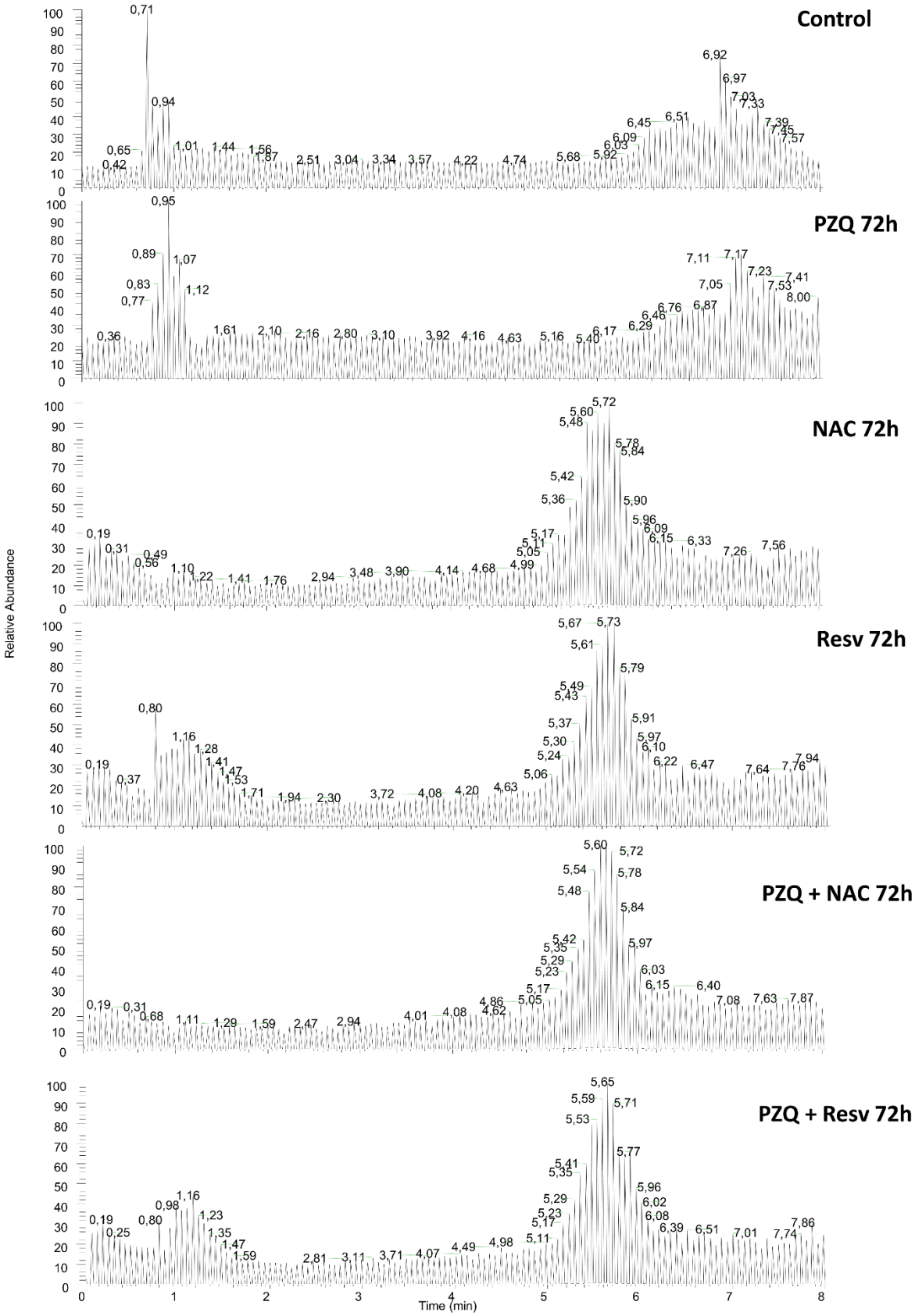
Figure S1. Representative micrographs of adult worms following 72h of exposure to anthelmintic [praziquantel (PZQ), artesunate (AS), flubendazole (FBZ)], anticancer drugs [imatinib (IMT), trametinib (TMT) and vandetanib (VDT)], antioxidants [(4-phenyl-1,2,5-oxadiazole-3-carbonile,2-oxide (OXA), *N*-acetylcysteine (NAC), resveratrol (Resv), flavone (Flav), H-Trp-Ser-OH (DiPept), melatonin (Mel)] alone and combined (1:1) at 100 μM.

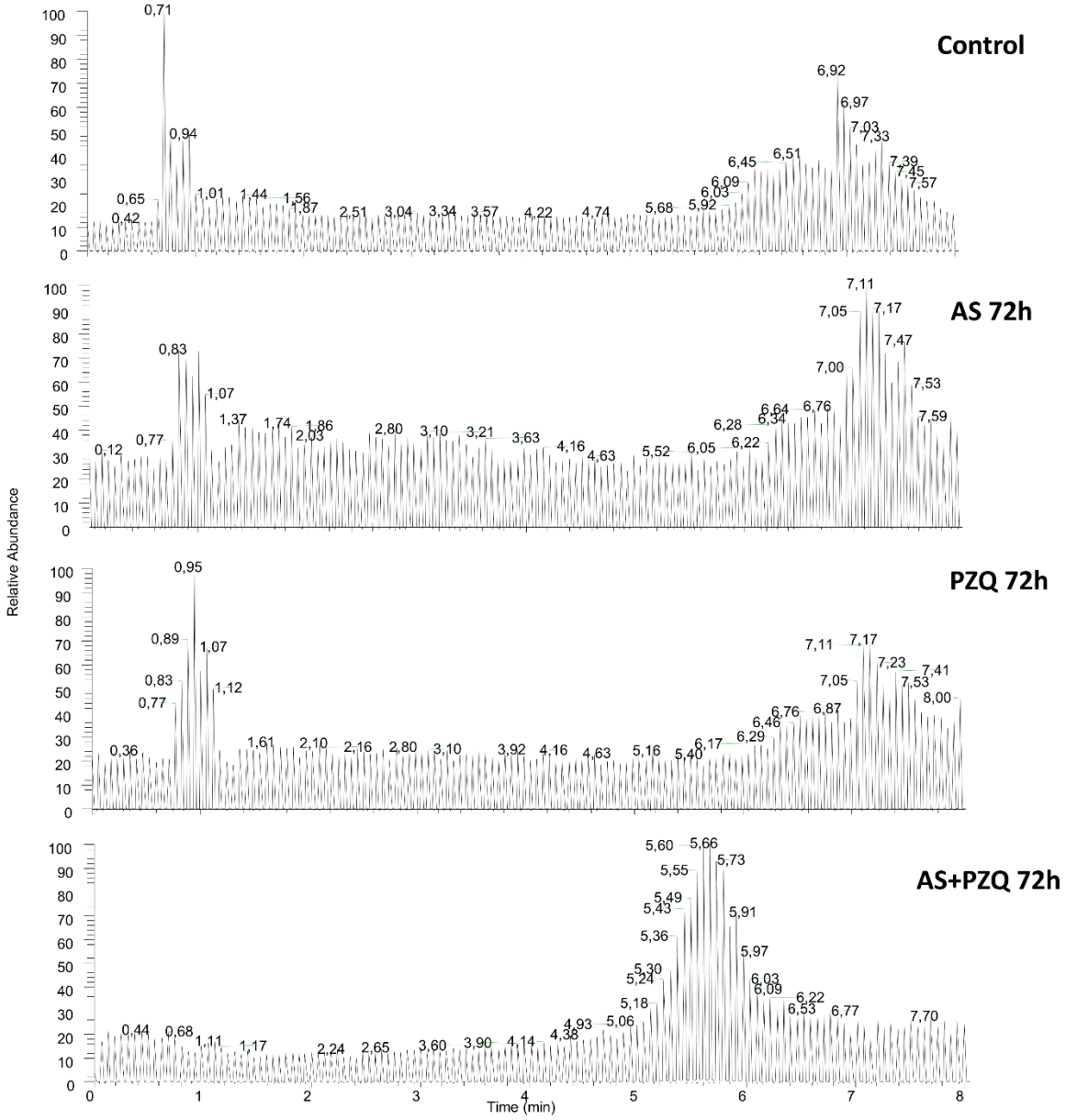
Appendix 6

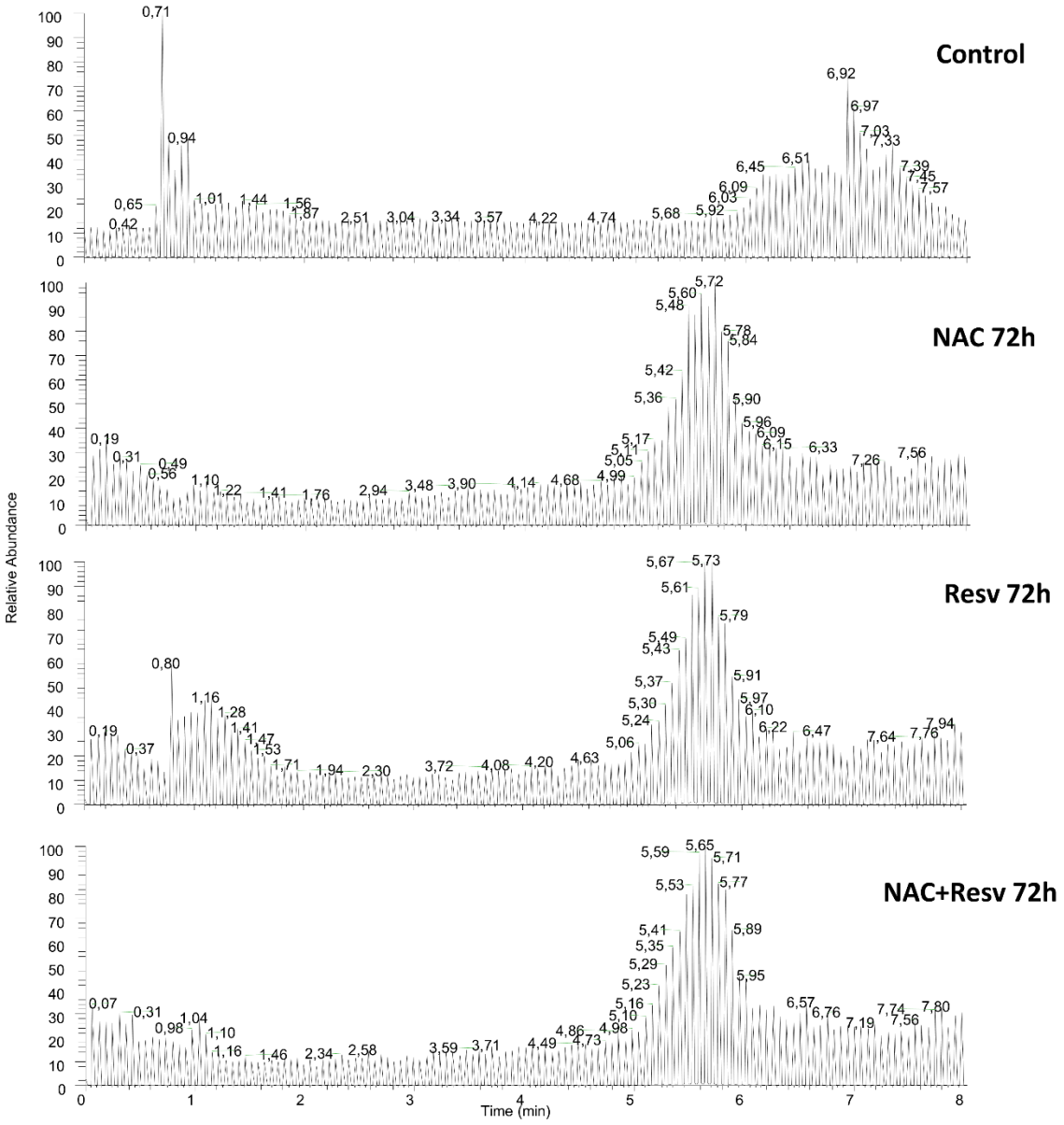
Support information of scientific article presented in Section III - Chapter 7

Figure S1: Mass spectra and m/z obtained for different samples analyzed by LC-MS/MS.




Control
PZQ 72h
NAC 72h
Resv 72h
PZQ + NAC 72h
PZQ + Resv 72h
Relative Abundance
Time (min)


Control
AS 72h
PZQ 72h
AS+PZQ 72h
Relative Abundance
Time (min)


Control
NAC 72h
Resv 72h
NAC+Resv 72h
Relative Abundance
Time (min)

Sample	m/z
Control (starting compounds plus enzymes)	184.00; 186.96; 188.02; 194.99; 195.02; 199.17; 204.00; 208.13; 208.98; 214.09; 217.18; 220.99; 225.01; 227.13; 240.99; 247.17; 257.98; 271.19; 279.16; 391.28; 319.00; 190.91; 226.95; 242.93; 258.90; 288.92; 294.94; 310.91; 326.89; **362.93**; **378.90**; 394.87; 430.91; 446.89; 462.86; 498.90; 514.88; 582.86; 650.85; 202.10; 218.94; 240.92; 256.90; 272.87; 332.89; **338.9**0; 354.88; 370.85; **414.9**0; **436.88**; **452.85**; 468.83; **474**.83; **512.79;** 534.85; **550**.83; **572**.81; **632.83**; **648.81**; 198.98; 220.26; 226.91; 256.90; 277.87; 278.88; 292.96; 294.85; 318.94; **346.80**; 354.88; 370.85; **376.86**; 390.93; 392.83; 410.90; 414.90; 452.85; 468.83; 474.83; **496.81; 594.78**; **610.77**; **746.78**; 198.98; 256.90; 278.88; 318.94; 332.89; 354.87; 376.86; 390.93; **416.91**; 430.87; 436.88; 452.85; 474.83; 488.91; **490.79;** 508.87; **588.79**; 606.85; **670.79**; 202.18; 276.98; 292.96; 312.92; 370.97; 374.96; 198.98; 256.90; 292.96; 390.94; **528.84**; **626.81**; **730.81; 768.70;** 214.94; 226.01; 466.32; **488**.30; 288.25; 391.28; **482.20**; 500.30; **522.29**
AS 72h	188.02; 194.99; 199.17; 204.00; 208.98; 209.01; 225.01; 240.99; 249.21; 257.98; 273.00; 279.16; 319.00; 391.28; 267.16; 190.91; 226.95; 242.93; 258.90; 294.94; 310.91; 326.89; **362.93**; **378.90**; 394.87; 430.91; 446.89; 462.86; 498.90; 514.88; 530.85; 582.86; 650.85; 180.90; 196.88; 232.92; 240.92; 242.92; 248.89; 256.90; 262.91; 264.86; 278.88; 284.89; 294.85; 300.86; 316.84; 316.88; 332.85; **338.90**; 368,85; 382.87; 398.84; 198.98; 220.26; 262,91; 272.87; 292.96; 318.94; 354.87; 370.85; **376.86**; 390.93; 410.91; **414.89**; **436.88**; **452.85**; **474.8**3; **490.79;** 495.81; **496.81; 550.83; 594.78; 610.77; 632.83;** 394.93; **416.91**; 488.91; 492.91; 508.87; **512.79; 588.79**; 606.85; **730.81**; **768.70**; 468.82; 276.98; 468.82; 374.96; 394.92; 312.92; **626.81**; 218.94; **528.84**; 390.94; 303.02; 257.98; 288.25; 307.15; 343.30; 345.17; 212.58; 268.16; 291.64; 466.32; 467.32; **488.30**; 256.30; 500.30; 304.30; **482**.29; **522.29**;
PZQ 72h	188.02; 194.99; 204.00; 208.98; 225.01; 240.99; 249.21; 257.98; 271.19; 273.00; 303.02; 313.19; 319.00; 180.90; 210.93; 223.06; 226.06; 232.92; 240.92; 242.92; 248.89; 262.91; 278.88; 284.89; 300.90; 310.91; 316.88; 352.88; 368.89; **436.88**; **452.85**; 196.88; 264.86; 300.86; 368.85; 382.86; 398.84; 420.82; 181.01; 220.96; 236.93; 256.90; 318.94; 354.87; 360.88; 370.97; **376.86**; **416.91**; 458.86; **474.8**3; 480.84; **496**.81; **512.79; 572.79**; **588.79**; **594.78**; **610.77**; **632.83**; **670.79; 746.78**; **768.70**; 226.91; 288.95; 390.93; 292.96; 198.98; 218.94; **338.9**0; 394.92; 410.90; 414.89; 508.87; 534.85; **550**.83; 276.98; 312.92; 374.96; 488.91; **490.79;** 606.85; **626.81**; 492.90; **528.84**; **648.81**; **730.81**; 390.94; 508.88; 466.32; 467.32; **488.3**0; 288.25; 500.30; **522.29**
NAC 72h	219.65; 220.15; 232.17; 240.16, 240.66, 279.16; 304.30; 340.26; 340.76; 359.23; 378.29; 378.79; 396.80; 443.27; 475.33; 588.41; 679.51; 701.49; 702.50; **338.90**; **378.79**; 397.30; 453.34; **490.79;** 283.72; 273.63; 415.77; 199.17; 214.09; 566.43; 184.00; 217.18; 213.02; 218.97; 273.17; 186.96; 188.02; 194.99; 204.00; 218.98; 223.96; 240.99; **346.87**; **572.79**; **588.79**; **648.81**; 981.41; 207.98; 260.95; 275.93; 306.26; 203.00; 225.20; 1327.91; 188.06; 190.14; 227.13; 981.40; 200.97; 391.28; 410.90; 198.34; 195.09; 199.03; 255.94; 793.35; 182.98; 239.07; 397.80; 453.84; 200.8; 200.24; 280.13; 301.14; 230.25;
Resv 72h	219.65; 220.15; 232.17; 240.16; 279.16; 304.30; 340.26; 340.76; 359.23; 378.29; 378.79; 396.80; 443.27; 475.33; 588.41; 589.41; 679.51; 701.49; 702.50; 199.17; 397.30; 415.30; 240.66; 283.72; 453.34; 566.43; 415.77; 184.00; 214.09; 217.18; 453.34; 453.84; 218.97; 273.63; 203.22; 220.93, 226.95; 814.58; 190.91; 242.93; 258.90; 294.94; 310.91; 326.89; **362.93**; **378.90**; 394.87; 430.91; 446.89; 462.86; **512.79;** 514.87; 530.85; **550.83**; **572.79**; 582.86; **594.78;** 598.84; **626.81**; 650.85; 717.47; **746.78**; **768.70**; 242.92; 240.92; 256.90; 262.91; 278.88; 292.96; 294.85; 300.86; 312.92; **338.9**0; 354.88; 360.88; **376.86**; 382.87; 398.84; 410.90; **436.88**; 458.86; **474.8**3; **496**.82; **588.79;** **610.77**; **670.79; 730.81**; 390.94; 394.92; **414.9**0; 430.86; **452.85**; 474.84; 508.88; 534.86; 196.96; 275.93; 332.89; **528.84**; 606.85; 626.82; **632.83**; 214.94; 218.98; 255.97; 332.88; 374.96; 223.96; 259.96; 260.95; 373.91; 234.91; 207.99; 204.00; 186.96; 275.92; 227.13; 264.99; 306.26; 391.28; 188.02; 188.06; 203.00; 225.20; 182.98; 200.97; 207.98; 213.02; 1327.96; 198.34; 188.06; 793.36; 680.52; 397.80; 453.85; 301.14; 230.25

m/z 466.32 correspond to glycocholic acid; m/z 313.19 correspond to PZQ.

Sample	m/z
AS+NAC 72h	199.17; 219.65; 279.16; 283.72; 304.30; 340.26; 340.76; 354.23; **378.90**; 396.80; 397.30; 453.34; 566.43; 679.51; 680.51; 701.49; 184.00; 214.09; 217.18; 415.77; 279.16; 190.14; 199.02; 213.02; 218.97; 255.94; 306.26; 188.02; 194.99; 204.00; 225.01; 225.20; 240.99; 184.00; 200.97; 273.63; 391.28; 186.01; 188.06; 203.00; 227.13; 527.78; **588.79; 670.79; 746.78; 768.70;** 182.98; 239.07; 397.80; 453.84; **512.79; 528.84;** 200.08; 301.14; 588.41; 280.16; 230.25;
AS+Resv 72h	1991.7; 219.65; 220.15; 232.17; 240.16; 279.16; 304.30; 340.26; 340.76; 359.23; 378.29; **378.79**; 396.80; 397.30; 443.27; 475.33; **588.41**; **670.79**; 679.51; 701.49; 702.50; 283.72; 453.34; 566.43; 214.09; 184.00; 217.18; 415.77; 213.09; 218.97; 306.26; 391.28; 1372.72; 186.96; 190.14; 204.00; 223.96; 255.94; 273.63; 203.00; 981.40; 188.06; 227.13; 186.01; 200.97; 225.20; 239.07; 215.16; 793.32; 1327.78; 397.80; 453.84; 200.08; 280.16; 301.14; 230.25: 284.33;
PZQ+NAC 72h	199.17; 219.65; 232.17; 240.16; 273.63; 279.16; 283.72; 340.26; 340.76; 359.23; 396.80; 397.30; 415.77; 443.27; 453.34; 566.43; **588.41**; 679.51; 701.49; 184.00; 214.09; 217.18; 218.97; **338.90**; 306.26; 453.84; **482.20; 496.81; 572.79; 594.78; 610.77; 632.83; 730.81; 746.78;** 186.96; 190.14; 213.02; 225.20; 255.94; 223.96; 793.31; 1327.79; 198.32; 203.00; 227.13; 793.30; 188.06; 204.00; 391.28; 200.97; 215.16; 981.40; 186.01; 215.16; 182.98; 239.07; 397.80; 680.52; 453.85; 488.30; 200.08; 200.24; 280.16; 301.14;
PZQ+ Resv 72h	219.65; 220.15; 232.17; 240.16; 279.16; 340.26; 340.76; 359.23; 378.29; **378.79**; 396.80; 415.30; 443.27; 475.32; 557.44; **588.41**; 679.51; 701.49; 702.50; 240.66; 283.72; 397.30; 453.34; 475.33; 199.17; 214.09; 217.18; 415.77; **474.83**; 566.43; 193.97; 226.95; 242.93; 604.38; 680.52; 717.47; 814.58; 190.91; 242.91; 258.90; 304.90; 310.91; 326.89; **362.93; 378.90**; 394.87; **436.80**; 446.89; 462.86; **488.30**; **496.81; 550.83; 670.79**; 718.47; 204.00; 220.93; 327.19; 491.30; 214.94; 240.92; 240.99; 255.97; 256.90; 262.91; 276.98; 278.88; 292.96; 312.92; **338.90**; 354.88; 370.97; 374.96; 390.94; 394.92; 410.90; 508.88; **414.90**; 430.86; **512.79; 528.84**; **572.79**; **594.78**; 606.85; **610.77; 626.81**; **746.78; 452.83**; **482.20**; 488.91; **522.29**; 218.98; 260.95; 275.93; 332.88; 223.96; 207.99; 259.96; 234.91; 188.02; 186.96; 207.98; 218.97; 194.99; 275.92; 306.26; 273.63; 391.28; 203.00; 200.97; 227.13; 213.02; 184.00; 198.33; 225.20; 793.34; 182.98; 188.06; 255.94; 397.80; 453.84; 466.32; 200.24; 200.08; 301.14; 313.19; 214.25;
AS+PZQ 72h	199.17; 219.65; 240.16; 279.16; 283.72; 301.14; 304.30; 340.26; 340.76; **346.80;** 352.34; 359.23; 396.80; 397.30; 401.29; 415.30; **416.91**; 445.31; 459.33; **482.20; 550.83; 588.41**; 679.51; 701.49; 273.63; 371.28; 443.27; 453.34; 214.09; 359.73; 415.77; 475.33; **488.30; 496.81**; 566.43; **594.78**; **648.81; 670.79**; **730.81; 746.78**; 214.09; 415.77; 566.43; 680.51; 217.18; 981.41; 184.00; 397.80; 186.96; 190.14; 213.04; 218.97; 306.26; 223.96; 203.00; 225.20; 255.94; 793.27; 1327.88; 188.06; 227.13; 198.32; 391.28; **768.70;** 793.29; 200.97; 195.09; 199.03; 527.78; 182.98; 215.16; 186.01; 453.84; 415.35; (313.14, pzq); 200.08; 200.24; 280.16; 230.25;
NAC+ Resv 72h	219.65; 220.15; 232.17; 240.16; 279.16; 301.14; 340.26; 340.76; 359.23; **378.29**; 396.80; 401.29; 415.30; **436.80**; 443.27, **452.85**; **482.20; 496.81; 528.84**; **588.41**, **626.81**; **632.83**; 679.51; 701.49; 702.50; 475.33; 589.41; 240.66; **416.91**; 453.34; 370.30; **376.86**; 397.30; 199.17; 283.72; 359.73; 214.09; 217.18; 415.77; 566.43; 184.00; 680.51; 188.02; 204.00, 214.94; 218.98; 223.96; 240.99; 255.97; 260.95; 275.93; 292.96; 312.92; 390.94; 410.90; 207.99; 218.97; 225.20; 306.26; 186.96; 203.0; 213.02; 227.13; 273.63; 391.28; 186.01; 198.34; 255.94; 793.36; 188.06; 200.97; 239.07; 182.98; 527.18; 397.80; 453.84; 475.32; 200.08;

Appendix 7

Spectrometric profiles liver flukes Opisthorchis and Fasciola provide insight into infection-induced carcinogenesis (submitted).

Spectrometric profiles between liver flukes *Opisthorchis* and *Fasciola* provide insight into infection-induced carcinogenesis

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Abstract

Chronic infections with the flatworm parasites *Opisthorchis viverrini*, *Clonorchis sinensis* and *Schistosoma haematobium* are classified as group 1 biological carcinogens, i.e. definitive causes of cancer. In addition, we reported findings that support the inclusion of *Opisthorchis felineus* in this list of biological carcinogens. By contrast, infections with close phylogenetic relatives including *Fasciola hepatica* have not been associated with carcinogenesis. Earlier reports revealed of oxysterol metabolites of *Opisthorchis* liver fluke origin conjugated with DNA bases, suggesting that the generation of these DNA-adducts may underlie the mutagenicity and carcinogenicity of the infection with these food-borne pathogens. Here we employed liquid chromatography-mass spectrometry (LC-MS/MS) to investigate, compare and contrast spectrograms of soluble extracts from *F. hepatica* adult worms from bile ducts of cattle with those from *O. viverrini* and *O*. *felineus* from experimentally-infected hamsters. *F. hepatica* and *Opisthorchis* spp. shared common compounds including oxysterol-like metabolites, bile acids and DNA-adducts, but the spectrometric profiles of *Fasciola hepatica* included far fewer compounds than *Opisthorchis species*. By this way, we can speculate why chronic infection with *F. hepatica* has not been associated with liver carcinogenesis. These findings support the postulate that oxysterol-like metabolites of parasite origin could be related to carcinogenesis associated to infection and they point to a molecular basis for the differences among major groups of liver flukes concerning infection-induced malignancy.

Author Summary

Several species of trematodes are parasites of the human hepatobiliary tract. Infection with two of these flukes, *Clonorchis sinensis* and *Opisthorchis viverrini*, freshwater fish-borne parasites that occur in East Asia is classified as group 1 carcinogens by the International Agency for Research on Cancer (IARC), i.e. definitive causes of cancer in humans. By contrast, infection with a different liver fluke, *Fasciola hepatica*, does not lead to malignant transformation of the biliary tract. Given the close phylogeny of all three parasites, this difference in carcinogenicity is intriguing and, if explained, likely of value in novel therapeutic approaches. The importance of the current findings is informative because they present a mass spectrometric analysis and

catalog of the similarities and differences between fluke of the genus *Opisthorchis* and *F. hepatica*, potentially identifying carcinogenic metabolites of liver fluke origin. These metabolites can be expected to provide deeper understanding of helminth infection induced malignancy.

Keywords

Fasciola hepatica, *Opisthorchis viverrini*; *Opisthorchis felineus*; oxysterols; DNA adducts

Introduction

More than 20% of cancer in the developing world are caused by infections [1]. The World Health Organization's International Agency for Research on Cancer (IARC) recognizes the infection with about 12 pathogens as group 1 biological carcinogens, i.e., definitive causes of cancer. These group 1 agents include three helminth parasites, specifically the fish-borne trematodes (FZT) *Opisthorchis viverrini* and *Clonorchis sinensis* and the blood fluke, *Schistosoma haematobium* [2]. In addition, we reported findings from hamster infection that support the inclusion of *Opisthorchis felineus*, also an FZT, to this list of biological carcinogens and definitive cause of cholangiocarcinoma [3]. We hypothesised that these helminths produce and release derivatives of oestrogens and oxysterols that promote oxidation of host DNA and have the ability of parasite metabolites to directly promote DNA lesions adducts and mutations that ultimately lead to cholangiocarcinoma [1,3-6]. The findings supported the postulate that these infection-associated cancers originate from a biological and/ or chemical insult followed by chronic inflammation, fibrosis, and a change in the tissue microenvironment that leads to a pre-cancerous niche [7,8]. Paradoxically, infections with other close phylogenetic relatives of these carcinogenic helminths, also food borne trematodes of the *Phylum Platyhelminthes* (Table 1), have not been categorized as group 1 biological carcinogens [9-15].

Table 1. Comparison of morphology, life cycle and pathogenesis between *Fasciola hepatica* and *Opisthorchis* species.

	 ***Fasciola* spp.**	 ***Opisthorchis* spp.**
MORPHOLOGY (ADULT WORM)	· Flatworms; leaf-like; 20-30 mm	· 5-10 mm
LIFE CYCLE	· Eggs embryonate in water releasing miracidia, which invade a suitable snail intermediate host. · Cercaria released from the snail encysts as metacercaria on aquatic plants. · After ingestion, the metacercaria excysts in the duodenum and migrates through the intestinal wall, the peritoneal cavity, and the liver parenchyma into biliary ducts, where development to the hermaphroditic adult stage occurs. · Starts laying eggs 3-4 months' post-infection. Long lived	· Eggs are ingested by freshwater snails release miracidia, which undergo several developmental stages in the snail. · Cercaria released form the snail encysts as metacercaria in fish. · After ingestion, the metacercaria excysts in the duodenum and ascends though the ampulla de Vater into the biliary tree where the liver fluke develops within the lumen of the bile ducts into the hermaphroditic adult form. · Starts laying eggs 3-4 weeks later. Long lived.
PHASES OF INFECTION	**Acute**: parenchymal stage; fluke migration into the bile duct where parasite ingests hepatic tissue; causes parenchymal destruction and immunologic and inflammatory reactions. **Chronic**: develops months after initial infection; consists of inflammation and hyperplasia of the epithelium and fibrosis; caused by the adult liver flukes residing in the bile ducts.	· Early stage no detectable change in the biliary epithelium and periductal areas of the liver. **Chronic**: desquamation of the biliary epithelium; epithelial hyperplasia; bile duct hyperplasia; periductal fibrosis; cellular infiltrates consist if lymphocytes, monocytes, eosinophils and some plasma cells; granulomatous inflammation; Histological analysis of liver sections from hamsters infected with *O. felineus* confirmed portal area enlargement, inflammation with severe periductal fibrosis and changes in the epithelium of the biliary tract characterized as biliary intraepithelial neoplasia, BilIN [3].
RELATION TO CANCER	· Unclear; no direct relationship has been definitively proved between the chronic infection and cancer. · Experimental data supporting opposing effects: **Tumor growth stimulation:** and proliferation of hepatocytes in acute phase of infection where juvenile flukes migrate through the parenchyma of the liver, provoking intense inflammatory response to antigens and secretory-excretory products from the flukes; induces proliferation of adjacent cells; accumulation of inflammatory cells capable of inducing DNA damage via ROS (ethene aducts) [9,10]. **Tumor inhibition:** observed in chronic phase of infection whereas acute infection may increase the metabolizing enzymes in the liver, and this decrease the activation of exogenous carcinogens, chronic infection reduces the metabolizing activity [11].	· Group 1 biological agent; infection is directly related to cholangiocarcinoma (CCA). · Postulated that *Opisthorchis* spp produces oxysterol-like metabolites that may act as initiators of the carcinogenesis associated to infection.
OTHER RELEVANT ASPECTS	Ectopic infections misdiagnosed as cancer; leads to increase of oxidative stress and lipid peroxidation; alters the antioxidant enzymatic system responsible to eliminate excess of ROS; mutability of CYP 2A5 [12].	

For instance, *Fasciola hepatica* has a wide geographical range, causes major economic loss in sheep and cattle worldwide, and also is an important food borne trematodes (FBT) pathogen of humans [16]. Despite fascioliasis can induce host DNA damage through action of reactive nitric species (RNS) or oxygen species (ROS) [15,17], however, the infection is not associated to carcinogenesis. Seeking new insights in the apparent paradox of differences in carcinogenicity among closely related FBZ, here we conducted an analysis of soluble extracts of adult worms of *F. hepatica, O. viverrini and O. felineus* by liquid chromatography coupled with mass spectrometry (LC-MS/MS). Remarkably, the LC-MS/MS chromatograms for each liver fluke species exhibited clear differences in regard the presence of oxysterols. These metabolites were minor components of the extract from *F. hepatica*, in contrast to the abundance and diversity of forms of oxysterols in *O. viverrini and O. felineus*. The presence of abundant oxysterols in the metabolites of *Opisthorchis* liver flukes support the notion that represent initiators of liver fluke infection-induced biliary tract malignancy.

Material and methods

Ethics Statement. Procedures undertaken complied with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm. Syrian hamsters (*Mesocricetus auratus*) were purchased from the stock of the Puschino Animal Facility (Russia) and bred at the Animal Facility of the ICG SB RAS (RFMEFI61914X0005) (Russia). The hamsters were maintained according to protocols approved by the Committee on the Ethics of Animal Experiments of the Institute of Cytology and Genetics (Permit Number: 25 of 12.12.2014).

Soluble extracts from *F. hepatica*, *O. viverrini* and *O. felineus* adult liver flukes. Adult worms of *F. hepatica* were obtained from the bile ducts of infected cattle at a local slaughterhouse [18]. It should be noted that the animals were processed as part of normal work of the slaughterhouse. *O. viverrini* and *O. felineus* were obtained as previously described [3,4]. In brief, metacercariae of *Opisthorchis* species were obtained from naturally infected cyprinoid fish in Khon Kaen province, Thailand or from

naturally infected fish (*Leuciscus idus*) in the Ob River near the city of Novosibirsk, Siberia Russia, respectively. The fish were digested with pepsin-HCl [3]. Fifty metacercariae were used to infect hamsters (*Mesocricetus auratus*) and three months after infection, the animals were euthanized and adult *O. viverrini* or *O. felineus* flukes recovered from their bile ducts. The worms were washed extensively in phosphate buffered saline (PBS, pH 7.4) supplemented with 100 µg/mL streptomycin and 100 U/mL penicillin G and cultured overnight in serum free RPMI-1640 medium (Lonza, Basel, Switzerland) containing 1% glucose, and protease inhibitors (0.1 mM phenylmethanesulfonyl fluoride, 2 µM E-64 and 10µM leupeptin) (Sigma-Aldrich, St. Louis, Missouri) at 37 °C, 5% CO_2.

Soluble extracts from all samples were prepared by sonication (5 x 5s burst, output cycle 4, Branson Sonifier 450, Germany) in PBS supplemented with protease inhibitors [500 µM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 0.3 µM aprotinin, 10 µM E-64, 10 µM bestatin and 10 µM leupeptin] (M221, Amresco, Solon, OH, USA), followed by 30 min centrifugation at 10,000 rpm, 4 ºC. The protein concentration of supernatants was determined using a commercial kit. Ascorbic acid was added to 1 mg/ml to these extracts, which were stored in aliquots at -80 ºC [3,4].

Sample preparation and LC-MS/MS analysis. Samples were prepared and processed using liquid chromatography diode array detection electron spray ionization mass spectrometry, as described [3-5]. Due to the acceptable chromatographic performance of methanol as the solvent in terms of separation and sensitivity, with short gradient times [19], this solvent was added up to 20% (v/v). High performance liquid chromatography coupled with mass spectrometer was employed to investigate molecular species from liver flukes, with samples of 25 µL injected into the LC-MS/MS instrument for analysis. The mass analysis was performed within an LTQ Orbitrap XL mass spectrometer (Thermo Fischer Scientific, Bremen, Germany), fitted with an ultraviolet (UV) photo diode array (PDA) detector. Analysis involved a Macherey-Nagel Nucleosil C18-column (250 mm x 4 mm internal diameter; 5 µm particle diameter, end-capped), proceeding at a flow rate of 0.3 ml/min. The capillary voltage of the electrospray ionization was 28 kW, capillary temperature was 310 ºC, flow rates of the sheath gas and auxiliary N2 were set to 40 and 10 (arbitrary unit as provided by the software settings), respectively, and gas temperature was 275 ºC [3-5]. The mobile

phase consisted of 1% formic acid in water (A)/acetonitrile (B) mixtures. Eluates were monitored for 75 min, run with a mobile phase gradient of 0-5 min, 100% A; 5-10 min, linear gradient from 100% to 80% A, 10-15 min 80% A, 15-50 min, linear gradient from 80% to 40% A; 50-65 min, 40% A; 65-75 min, linear gradient from 40% to 100% B. Washing for 15 min with acetonitrile was carried out to stabilize the column. Data were collected in negative electrospray ionization negative mode scanning a mass to charge ratio (m/z) range of 50-2000.

Results

Both species of *Opisthorchis* shared identical mass spectra profiles.

We have developed a sensitive LC-MS/MS-based protocol to identify new steroids-derived molecules not only in extracts of helminth parasites [3,4], but also from experimental infected rodents [4] and naturally-infected humans [5]. Extracts obtained from *F. hepatica* adult worms were analyzed in order to provide insights related to their composition and complexity.

Comparing data obtained for *O. viverrini* with *O. felineus* we observed that both these liver flukes displayed highly similar mass spectra (MS) and shared most peaks detected (indicated in grey in Fig 1) which were attributed to oxysterol-like metabolites, e.g. mass/charge (m/z) 356, 307, bile acids in oxidized form, e.g. m/z 443, 479, 488 and DNA-adducts, e.g. m/z 599, 639, 667 [3,4].

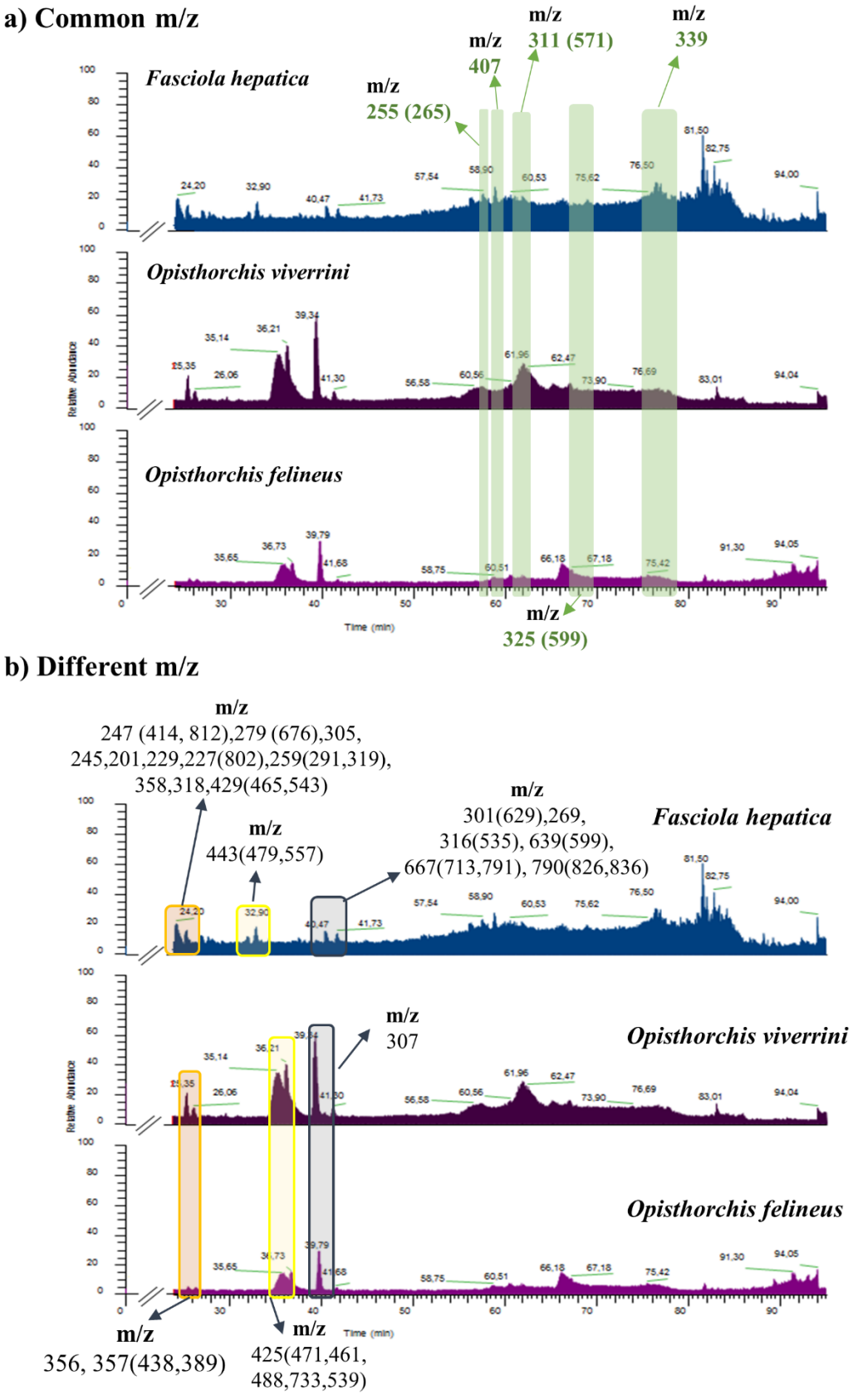

Fig 1. Comparison of mass spectral profiles obtained for *Fasciola hepatica* and *Opisthorchis* spp. Panel A, common m/z between the three liver flukes; panel B, major differences among the liver flukes.

F. hepatica extracts exhibited striking differences to those of the _Opisthorchis_ species.

Notable differences were apparent among the MS profiles of *F. hepatica* and the *Opisthorchis* species. Most of compounds present in both *Opisthorchis* species were absent from *F. hepatica*, specifically m/z 356, 357, 425 and 307. Remarkably, these specific compounds were attributed to be oxysterols with ability to react with host DNA as described [3]. The MS profile of *F. hepatica* was much more complex than those obtained for *Opisthorchis* spp. (Fig 1). The major differences were observed at retention intervals of approximately 24, 32, and 40 min – as indicated in orange, yellow and blue, respectively, on the chromatographs (Fig 1). On these retention times, *F. hepatica* showed greater number of compounds in comparison to those observed on *Opisthorchis* species (Fig 1 and Table 2). Remarkably, most of these compounds were detected only in *F. hepatica* extracts (Table 2).

Unlike *Opisthorchis*, *F. hepatica* displayed more compounds with elevated m/z (between 600 and 800), mostly between retention interval of 38 to 42 min (Table 2), which might suggest that they are more complex than the majority of those detected on *Opisthorchis* spp.

Nonetheless, *F. hepatica* and *Opisthorchis* spp. shared several common compounds at retention interval of 58-64 min (signed by green in Fig 1 and Table 2). These compounds have been ascribed previously to oxysterol-like metabolite (e.g. m/z 325), bile acids (e.g. m/z 571) and as well as DNA-adducts (m/z 599) [4]. To reiterate, however, these were fewer of these compounds in *F. hepatica* compared to *Opisthorchis* spp.

Table 2. Comparison of mass/charge (m/z) obtained for *Fasciola hepatica* during this study with *Opisthorchis* spp. previously reported for *O. viverrini* [3,4] and *O. felineus* [3]. The structures of common m/z (signed at green) are depicted on S1 Table.

Retention time (min)	m/z	*Fh*	*Ov*	*Of*
17.64	337.08	✓	✓	
23.24	320.17	✓		
23.25	353.13	✓		
23.95	293.12	✓		
24.17	245.12	✓		
	414.22	✓		
24.26	279.14	✓		
	676.30	✓		
24.36	305.08	✓		
24.41	245.12	✓		
24.75	229.16	✓		
	201.13	✓		
25.08	227.14	✓		
25.13	259.13	✓		
	291.10	✓		
	319.10	✓		
25.41	358.20	✓		
25.54	318.11	✓		
28.12	429.23	✓		
	465.20	✓		
	543.22	✓		
32.89	443.24	✓		
37.65	301.07	✓		
38.18	629.30	✓		
39.50	316.17	✓		
	535.23	✓		
40.50	677.50	✓		
	713.48	✓		
	724.51	✓		
41.70	790.58	✓		
	826.56	✓		
	837.59	✓		
51.02	447.14	✓		
54.86	321.18	✓		
58.70	255.23	✓	✓	✓
58.92	407.28	✓	✓	✓
59.93	571.29	✓	✓	✓
61.06	311.17	✓	✓	✓
64.25	325.19	✓	✓	✓
64.15	599.32	✓	✓	✓
70.16	367.25	✓		
77.95	339.20	✓	✓	✓
76.38	391.29	✓		
81.63	465.31	✓		

Discussion

Both chronic infection with *Fasciola* spp. and *Opisthorchis* spp. could lead to fibrosis, hyperplasia and biliary stasis [3,10,21-23]. However, an association between fascioliasis and cancer remains controversial and not definitely established [10]. Thus, we decided to investigate extracts of adult worms of *F. hepatica* and compare with data previously obtained for *Opisthorchis* spp. We aimed to address the following questions: 1) does *F. hepatica* synthesize and excrete metabolites that promote direct damage on host DNA, and 2) if *F. hepatica* induces DNA damage, why has fascioliasis not been associated with liver cancer in ungulates or indeed humans? The MS profile of *F.* hepatica was found to be far more complex, showing an elevated number of compounds with an elevated m/z rather than *Opisthorchis* spp. This suggested that metabolic process that occur in *F. hepatica* are dissimilar to those in *Opisthorchis* spp.

The LC-MS/MS analysis also revealed a great diversity of compounds with different m/z. This diversity might reflect fragmentation of a number of compounds, detected here as lower m/z fragments of other compounds. However, we cannot conclude that these are not novel compounds. In addition, some of these compounds might be precursors of known compounds recorded previously [3,4]. Compounds of *F. hepatica* might be related to the different migratory route of the parasite to the biliary tree. Unlike *Opisthorchis* spp., newly excysted juveniles of *F. hepatica* exit the lumen of the small intestine, transverse the intestinal wall and migrate through the abdominal cavity to the Glisson´s capsule of the liver [20,24]. This parasite might deploy more complex biochemical processes and secretions, including the secretion of cathepsins [25-27] to accomplish this elaborate organ and tissue migration. The juvenile *F. hepatica* infects the liver by directly penetrating the Glisson's capsule from the abdominal cavity, and thereafter burrows through the hepatic parenchyma to the bile ducts where it eventually matures into the egg-laying adult worm (S1A Fig) [20]. Components detected in the extracts of *F. hepatica* might be related with digestion of host tissues including blood such as hemoglobin, albumin and immunoglobin to support reproductive process including synthesis of eggs [20]. This might not only explain the complex MS profile but also the compounds with elevated m/z as well as lower m/z that could be associated with free amino acids. On other hand, most of the compounds observed from 23 to 57 minutes were specific of *F. hepatica*, i.e. not present in *Opisthorchis*.

Juvenile *Opisthorchis* flukes ascend from the duodenum directly into the lumen of biliary tree [23,28].

Glycocholic acid in the mammalian small intestine triggers the excystment of the metacercaria and emergence of *F. hepatica* juvenile flukes stimulating the exit of the parasite from the gut lumen and its migration to the abdominal cavity. Intriguingly, the juvenile *F. hepatica* did not survive in bile-containing solutions whereas the adult fluke resides in the bile ducts, bathed in bile [29]. Differences in the nature of the juvenile versus adult tegument of *F. hepatica* and the selectivity and the permeability of glycocalyx of the tegument may underpin these stage specific differences [29]. The complexity of the tegument, a complex metabolically active and highly glycosylated biological matrix [30] might also underpin complexity of *F. hepatica* MS profile and its components.

Both *F. hepatica* and the two *Opisthorchis* species shared some identical compounds that were previously attributed to oxysterol-like metabolites, bile acids and DNA-adducts. This is feasible since all three flukes live within the biliary tree. There is evidence that *F. hepatica* induces DNA damage through the action of mutational-mediators [9,31]. The presence of DNA adducts in tissue does not necessarily imply a specific tumorigenic risk for the host tissue. Other factors such as DNA repair and cell proliferation key roles players in determining the overall carcinogenic risk [32]. An association between fascioliasis and cancer has only been suggested from *in vitro* studies and, thus far, there have not been satisfactory reports of human cases of bile duct cancer due to chronic infection with *F. hepatica* [10,14,33-35]. Therefore, there is a lack of cogent evidence that relate fascioliasis with cancer [10]. By contrast, a number of reports posit opposing effects, i.e. tumor growth stimulation and inhibition. Tumor growth stimulation and proliferation of hepatocytes has been observed during acute phase of infection where larval flukes migrate through the parenchyma of the liver and provoke marked inflammation [12,17]. In turn, the chronic inflammation increases oxidative stress that can overwhelm antioxidant system homeostasis to dampen reactive oxygen species and consequent oxidative modification of lipids, nucleic acids and proteins [9]. Like fascioliasis, opisthorchiasis is characterized by elevated oxidative stress and altered the antioxidant systems [9,11]. Tumor inhibition has been noted during the chronic phase of fascioliasis that may dampen the liver metabolizing activity [12]. We also documented that infection with *O. felineus* induces BilIN. The

consonance of findings that the presence of new metabolites and of BilIN-1 and BilIN-2 indicates that *O. felineus* infection induces neoplastic transformation of cholangiocytes and can be expected to promote growth of biliary cancers [3]. Whereas acute *F. hepatica* infection may increase the metabolizing enzymes in liver and thus increase the activation of exogenous carcinogens [22], chronic infection may reduce hepatic metabolizing activity [12]. It is noteworthy that chronic infection with *F. hepatica* in a rat model suppressed *N*-nitrosodimethyldiamine-induced carcinogenesis, suggesting a parasite-induced inhibition of carcinogenesis in the liver of rodents experimentally infected with *F. hepatica* [17]. All these hypotheses require further investigation and experimental validation. This study aimed to characterize the differences between *Fasciola* and *Opisthorchis* with a view to identifying the parasite-derived compound that results in the different pathogenic outcomes after infection with these two parasites. Not only is this important to understanding the mechanism of pathology, with a view to perhaps developing appropriate therapeutics in the future, but it would also provide information to understand how two flukes have adapted to induce such different outcomes in their hosts.

Fasciola hepatica displayed more complex mass spectra profile that the *Opisthorchis* species and several specific compounds that might be related to its complex route of migration to the biliary tract. Nonetheless, *F. hepatica* shared several compounds with *Opisthorchis,* which are related to oxysterols, bile acids and DNA-adducts. The presence of only a few common compounds might explain why fascioliasis has not been causally linked with liver cancer. Indeed, we posit that fewer oxysterol-like metabolites might (partially) explain why definitive carcinogenic potential has not been ascribed to ruminant or human fascioliasis (S1B Fig). On the other hand, it has been shown that *F. hepatica* could suppress reactivity of liver carcinogens.

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Declarations of conflicting interests

The authors declare no conflicts of interest with respect to the research, authorship, and/or publication of this article.

Supporting information

S1 Table. Structures of m/z common to *Fasciola hepatica* and *Opisthorchis* species.

S1A Fig. Different routes that liver flukes undergo to reach the biliary tree. *F. hepatica* (signed at blue) transverses the intestinal wall and migrates through peritoneum to the Glisson´s capsule of the liver, perforate the capsule enters the liver parenchyma and migrates to the biliary tree. In contrast, Opisthorchis spp. juveniles pass through the stomach to the duodenum with ingested fish, after which they ascend into the biliary tract through the ampulla de Vater (signed at yellow). This might constitute the major reason for complexity of mass spectra profile of *F. hepatica*.

S1B Fig. Adult liver flukes *O. viverrini* and *O. felineus* produces oxysterol-like metabolites that interact with host chromosomal DNA to form DNA-adducts and forms of biliary intraepithelial neoplasia that conducive to cholangiocarcinoma. *F. hepatica* also elaborates oxysterol-like metabolites, but at much lower number, which might be explain, at least in part, why infection with this parasite fails to induce malignancy.

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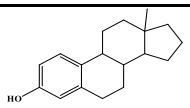
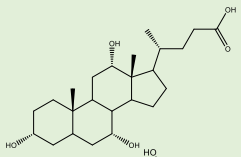
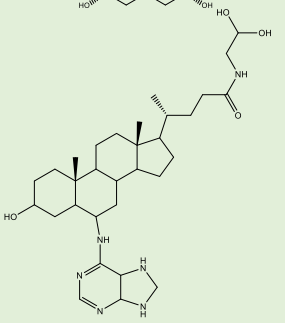
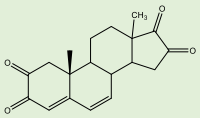
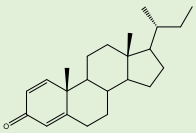
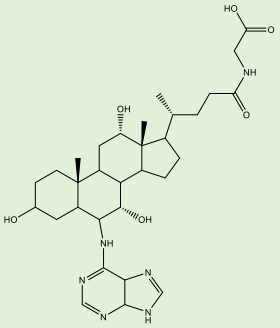
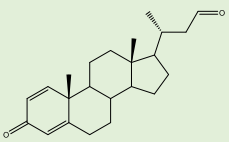
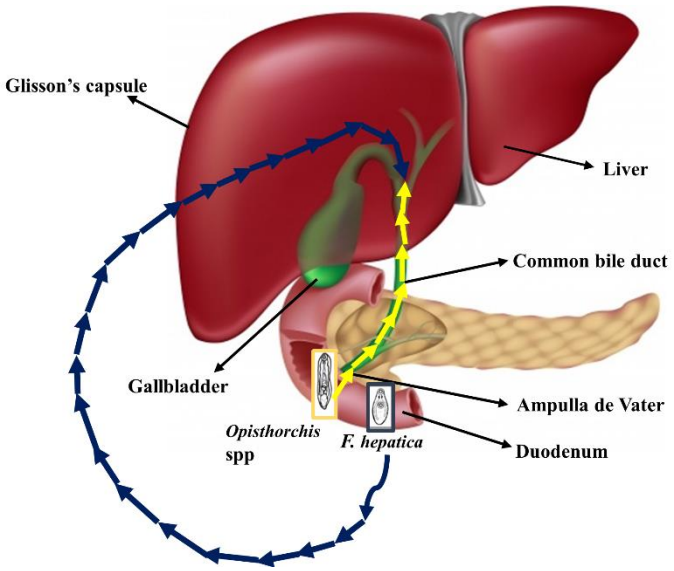
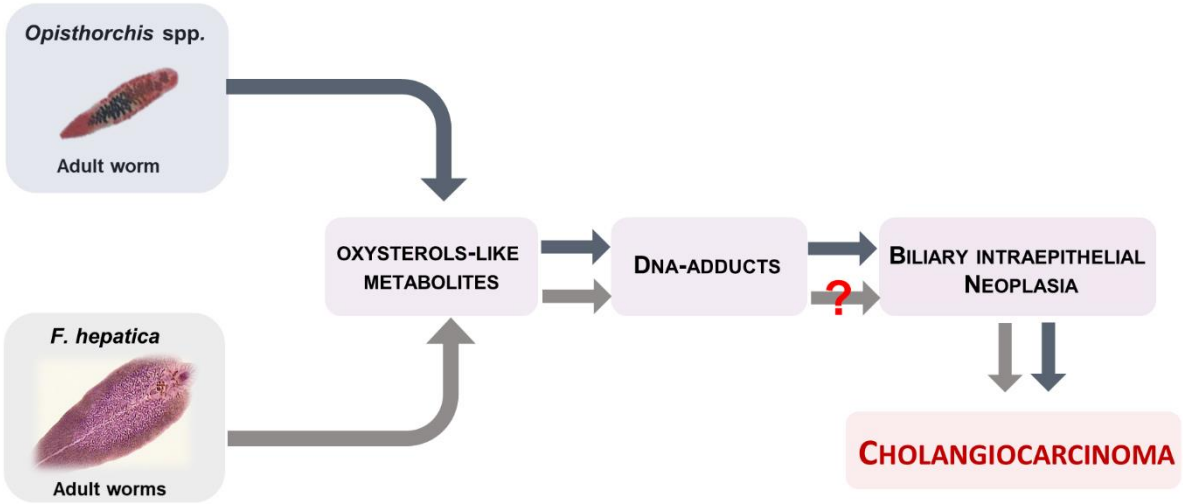
Supplementary Table S1. Structures of m/z common to *Fasciola hepatica* and *Opisthorchis* species.

Retention time (min)	m/z	*Fh*	*Ov*	*Of*	Structures
57.54	255.07	✓	✓	✓	
58.92	407.28	✓	✓	✓	
59.93	571.29	✓	✓	✓	
61.06	311.17	✓	✓	✓	
64.25	325.19	✓	✓	✓	
64.15	599.32	✓	✓	✓	
77.95	339.20	✓	✓	✓	



S1A Fig. Different routes that liver flukes undergo to reach the biliary tree. *F. hepatica* (signed at blue) transverses the intestinal wall and migrates through peritoneum to the Glisson´s capsule of the liver, perforate the capsule enters the liver parenchyma and migrates to the biliary tree. In contrast, *Opisthorchis* spp. juveniles pass through the stomach to the duodenum with ingested fish, after which they ascend into the biliary tract through the ampulla de Vater (signed at yellow). This might constitute the major reason for complexity of mass spectra profile of *F. hepatica*.



S1B Fig. Adult liver flukes *O. viverrini* and *O. felineus* produces oxysterol-like metabolites that interact with host chromosomal DNA to form DNA-adducts and forms of biliary intraepithelial neoplasia that conducive to cholangiocarcinoma. *F. hepatica* also elaborates oxysterol-like metabolites, but at much lower number, which might be explain, at least in part, why infection with this parasite fails to induce malignancy.